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Transcriptional regulatory network analysis and its application for increasing antibiotics production in *Streptomyces coelicolor*

방선균에서 전사 조절 네트워크 분석 및 항생제 생산 증가에의 활용

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서울大學校 大學院

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李 보 람

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Transcriptional regulatory network analysis and its application for increasing antibiotics production in *Streptomyces coelicolor*

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By

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Abstract

Transcriptional regulatory networks in *Streptomyces coelicolor* A3(2) have evolved to execute various cellular processes affecting antibiotic production. Several key transcriptional regulators involved in biosynthesis of secondary metabolites have been characterized, including ActII-ORF4, RedD, and CdaR. However, new regulators are still needed to be identified to understand the complex transcriptional regulatory networks delineating antibiotic production. Here, we identified a list of regulators controlled by AbsB acting as a higher level regulator in the antibiotic production. These potential regulators were identified to show different expression patterns in the absence of *absB* gene. RNase cleavage assay revealed that *sco6808* transcripts, one of the potential regulators, are cleaved by AbsB. This result indicates that *sco6808* is a direct target of AbsB. Therefore, our approach provides a comprehensive list of novel regulators controlled by AbsB, which serves as a basis for understanding multi-layered regulatory networks.

Manipulation of multiple transcription regulators controlling non-redundant pathways related to antibiotics production would effectively increase the antibiotics production. Here, we present an approach that uses a combination of two transcriptional regulators that control independent pathways to increase the antibiotics production. AfsS is one of key master activators of antibiotics production. Using the microarray data of *afsS* disruption mutants, we first selected the regulators transcriptionally independent of *afsS*. Among them, we focused on *sco4677*, an antagonistic

regulator of sigma factor F, and *sco4228* (*phoU*), a phosphate transport system regulator. Using the combination of *sco4677* with *afsS* (BG4677S) and *phoU* with *afsS* (BG4228S), we observed the intracellular actinorhodin production increased in BG4677S by approximately 11-folds higher and BG4228S by approximately 149-folds higher than wild type.

Time-course microarray experiments were performed using *ndgR* deletion mutants for expanding the fragmented transcriptional regulatory network. Previously, NdgR was identified by DNA affinity capture assay (DACA) and mass spectrometric analysis as a global regulator that controls amino acids synthesis, quorum sensing and antibiotics production. In addition, we unveiled that NdgR also controls ABC transport system of glutamate, branched chain amino acid, phosphate and methionine, carbohydrate uptakes, carbohydrate metabolism in PTS, glycerol metabolism, cell stress response, and tolerance to toxic materials. Those roles were confirmed by EMSA, cell viability test, and the observation by confocal microscope and TEM images. From these results, we could accumulate the information about the role of the global regulator, NdgR, to the incomplete transcriptional regulatory network in *Streptomyces coelicolor*.

In conclusion, we attempted to identify the transcriptional regulatory network and developed a capable method to increase the antibiotics production.

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Key words: *Streptomyces*, transcriptional regulator, mass spectrometry, DNA chip, regulatory network, antibiotics overproduction

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Chapter I

Introduction

1.1 Regulations in *Streptomyces*

Streptomyces, a representative Gram-positive soil bacterium, has complex life cycle and secondary metabolism to produce various antibiotics and natural products. Since this microbe is the most well-known bacterium producing most of industrial antibiotics and natural products used as medicine, correct understanding of their biosynthetic control mechanisms and their networks is a long time-awaited issue in pharmaceutical industry. Control of biosynthetic pathways of secondary metabolites takes place through various cellular events, such as transcription, translation, post-translation, nutrient and precursor supply and uptake, transporter regulation and secretion. Among them, transcriptional regulation is the primary and coarse regulation of the gene expression of biosynthetic gene clusters. Such transcriptional regulation is controlled at multi-levels via complex transcriptional network.

1.1.1 Regulatory networks studied in *Streptomyces*

It is common knowledge that streptomycetes have complicated transcriptional regulatory networks because of their complex life cycle and secondary metabolism. Over the decades, people have made efforts to understand transcriptional regulatory networks to control the cellular metabolisms.

One of the well-known transcriptional regulatory networks is focused on the antibiotics cluster regulation. StrR, a streptomycin production activator (Vujaklija D Fau - Horinouchi *et al.*, 1993) and ArpA, an A-factor receptor as a repressor (Onaka *et al.*, 1995, Onaka & Horinouchi, 1997) was first studied in *S. griseus*. ActII-ORF4, an activator

of actinorhodin production (Gramajo *et al.*, 1993), RedD, an activator of undecylprodigiosin production (Feitelson *et al.*, 1985, Narva & Feitelson, 1990) and CdaR as an activator of calcium-dependent antibiotic (CDA) production (Ryding *et al.*, 2002) have been studied in *S. coelicolor*. Globally influenced regulators have been also studied in streptomycetes. AbsB, encodes an RNase III homologue, affects various target genes such as antibiotics production regulators as well as morphological developmental regulators (Price *et al.*, 1999, Xu *et al.*, 2008, Xu *et al.*, 2010c). Regulators which are related with the nutrient starvation and stringent response are known their functions. It is that PhoR/PhoP regulatory networks are responded by the concentration of phosphate (Sola-Landa *et al.*, 2003, Santos-Beneit *et al.*, 2009, Sola-Landa *et al.*, 2005, Mendes *et al.*, 2007, Sola-Landa *et al.*, 2008). Moreover, PhoR/PhoP regulatory systems are linked with the two-component regulatory network of AfsK/AfsR/AfsS (Lee *et al.*, 2002, Tanaka *et al.*, 2007, Horinouchi *et al.*, 1990, Tomono *et al.*, 2006) and the nitrogen response regulatory network of GlnR/GlnA (Lewis *et al.*, 2011, Martin *et al.*, 2011).

Carbon catabolism and carbon uptake systems are also essential regulatory systems for the antibiotics production. Among them, N-acetyl glucosamine (GlcNAc) related regulatory system has been revealed recently. DasR is a major regulator of GlcNAc uptake and GlcNAc metabolism (Rigali *et al.*, 2006, Rigali *et al.*, 2008, Nothaft *et al.*, 2010). DasR controls phosphoenolpyruvate-dependent phosphotransferase system (PTS) and metabolic enzymes such as NagA/NagB/NagK by repression of target gene expressions and DasR is affected by glucosamine-6-P (GlcN-6-P) as an .intermediate molecule of GlcNAc.

Table 1.1 Regulators related with antibiotics production in *Streptomyces*. Adopted and modified from (van Wezel & McDowall, 2011)

Gene/ ID	Functions of regulators	reference
Regulators with ungapped links that extend beyond antibiotic gene clusters		
strR/ SGR5931	Ultimate (final) regulator of the streptomycin cluster in *S. griseus*; transcriptional activator	(Vujaklija D Fau - Horinouchi et al., 1993)
adpA/ SGR4742	Activator of *strR*; *S. coelicolor* homologue is BldH; member of AraC/XylS family	(Ohnishi Y Fau - Kameyama *et al.*, 1999)
arpA/ SGR3731	Transcriptional repressor of *adpA*; receptor for A-factor, which blocks DNA-binding activity	(Onaka et al., 1995, Onaka & Horinouchi, 1997)
afsA/ SGR6889	Catalyses committal step in synthesis of A-factor; *S. coelicolor* homologue is ScbA	(Kato *et al.*, 2007)
arfA/ SCO3841	Binds to promoters of *adpA* (*bldH*) in both *S. griseus* and *S. lividans*; evolutionarily conserved; disruption reduces level of undecylprodigiosin (RED) produced in *S. lividans*; lacks an obvious DNA-binding domain	(Xu *et al.*, 2009)
actII-ORF4/ SCO5085	Ultimate activator of the ACT (act) biosynthetic cluster in *S. coelicolor* and *S. lividans*; SARP family regulator	(Gramajo et al., 1993)
redD/ SCO5877	Ultimate activator of the RED (red) biosynthetic cluster in *S. coelicolor* and *S. lividans*; SARP family regulator	(Feitelson et al., 1985, Narva & Feitelson, 1990)
dasR/ SCO5231	Transcriptional repressor of *actII-ORF4*; receptor for GlcN-6-P, which blocks DNA-binding activity; master regulator of genes involved in chitin catabolism; links to development; regulator of *scbA*	(Rigali et al., 2008, Rigali et al., 2006, Nothaft et al., 2010)
atrA/ SCO4118	Transcriptional activator of *actII-ORF4*; member of TetR family; transduced signal is unknown; homologue of AveI in *S. avermitilis*; conditional regulator of *S. griseus strR*; activator of *nagE2*	(Hirano *et al.*, 2008, Uguru *et al.*, 2005, Nothaft et al., 2010, Chen *et al.*, 2008)
rok7B7/ SCO6008	Transcriptional activator of *actII-ORF4*; regulator of xylose metabolism; regulon overlaps that of DasR	(Park *et al.*, 2009)
absA1/2/ SCO3225/3226	Two component system; located in calcium-dependent antibiotic (CDA) cluster; negatively regulates CDA production, regulation appears independent of CdaR; AbsA2 binds to *actII-ORF4* increasing Act production; transduced signal is unknown; reported to bind to promoter of *redD*	(McKenzie & Nodwell, 2007, Sheeler *et al.*, 2005, Anderson *et al.*, 2001, Ryding et al., 2002)

slpR/ SCO0608	Binds to the promoters of *actII-ORF4* and *redD*; negative regulator of antibiotic production and sporulation in *S. coelicolor*	(Yang *et al.*, 2008)
SCO6808	Binds to the promoters of *actII-ORF4* and *redD*; negative regulator of antibiotic production and sporulation in *S. coelicolor*	(Yang et al., 2008)
redZ/ SCO5881	Orphan response regulator; transcriptional activator of *redD*; TTA codon in gene explains *bldA* dependence of RED production	(Guthrie *et al.*, 1998)
cpkO/ SCO6280	Ultimate activator of the cryptic polyketide (CPK) cluster in *S. coelicolor*; gene also called *kasO*	(Pawlik *et al.*, 2007)
scbR/ SCO6265	Transcriptional repressor of *cpkO*; DNA-binding activity blocked by gamma-butyrolactones; autoregulates own expression; located within cpk cluster; impacts ACT production	(Takano *et al.*, 2001)
scbA/ SCO6266	Catalyses committal step in synthesis of *S. coelicolor* gamma-butyrolactones; *S. griseus* homologue is *afsA*; transcription activated by ScbR; located within cpk cluster; impacts ACT production	(Takano et al., 2001)
ndgR/ SCO5552	Binds to the promoter of *scbR*; controls leucine biosynthesis; activity dependent on nitrogen; widely conserved in streptomycetes; member of IclR family; homologue regulates doxorubicin in *S. peucetius*	(Yang *et al.*, 2009)
relA/ SCO1513	Under limited amino acids or glucose, catalyses synthesis of (p)ppGpp, which binds RNA polymerase and signals start of stringent response; essential for antibiotic production in *S. coelicolor* and other species	(Chakraburtty *et al.*, 1996, Ryu *et al.*, 2007, Chakraburtty & Bibb, 1997)
rshA/ SCO5794	Catalyses (p)ppGpp synthesis under conditions of limited phosphate	(Sun *et al.*, 2001, Ryu et al., 2007)
relC/ SCO4648	Also called RplK; specific mutation impairs production of (p)ppGpp and ACT in *S. coelicolor*; confirmed in other species	(Ochi, 1990a, Ochi, 1990b, Kelly *et al.*, 1991)
rpoB/ SCO4654	Subunit of RNA polymerase; mutants that confer rifampicin resistance confer stringent response-like characteristics	(Hosaka *et al.*, 2009, Lai *et al.*, 2002)
cprA/ SCO6071	Encodes ArpA homologue that activates antibiotic production	(Onaka *et al.*, 1998)
cprB/ SCO6312	Encodes ArpA homologue that is 91% identical to CprA, but represses antibiotic production	(Onaka et al., 1998)
SCO3201	TetR homologue; represses antibiotic production when placed on multi-copy vector; no clear phenotype for deletion mutant	(Xu *et al.*, 2010a)

Regulators in pathways with missing link to antibiotic gene clusters		
afsS/ SCO4425	Also called AfsR2; similar to sigma factors; overexpression stimulates antibiotic production in several species; regulator of nutrient stress responses	(Lee et al., 2002, Tanaka et al., 2007, Vogtli *et al.*, 1994, Lian *et al.*, 2008)
afsR/ SCO4426	Similar to SARPs; activates transcription of *afsS*; DNA-binding activity blocked by phosphorylation	(Floriano & Bibb, 1996, Horinouchi et al., 1990, Horinouchi, 2003)
afsK/ SCO4423	Major serine/threonine kinase that phosphorylates AfsR; shown to bind AdoMet	(Matsumoto *et al.*, 1994, Umeyama *et al.*, 1999, Tomono et al., 2006)
kbpA/ SCO4422	Modulates ability of AfsK to phosphorylate AfsR	(Umeyama & Horinouchi, 2001)
pkaG & *afsL*/ SCO4487, SCO4377	Two AfsR-like kinases that also phosphorylate AfsR	(Sawai *et al.*, 2004, Horinouchi, 2003)
phoR/P/ SCO4229, SCO4230	Two component system that regulates uptake and assimilation of phosphate; perturbation of system stimulates antibiotic production in *S. coelicolor* and *S. lividans*; PhoP binds promoter of AfsS probably blocking activation by AfsR; PhoP also binds promoter of *glnR*, a major regulator of nitrogen assimilation	(Sola-Landa et al., 2003, Santos-Beneit et al., 2009, Sola-Landa et al., 2005, Mendes et al., 2007, Sola-Landa et al., 2008)
bld	Various Several regulatory proteins required for the development of aerial hyphae from the previously 'bald' vegetative mycelium, as well as for production of antibiotics	(Chater, 2006, Flardh & Buttner, 2009, Kelemen & Buttner, 1998, Chater, 2001)
dmdR1/adm/ SCO4394	Regulators of iron metabolism; *adm* overlaps dmdR1 on the opposite strand; disruption of *adm* increases production of ACT and RED	(Tunca *et al.*, 2009, Tunca *et al.*, 2007)
facC/ AF103943	Factor C; secreted signalling protein that stimulates sporulation and has a link to A-factor production; extracellular Factor C induces submerged sporulation and streptomycin production in *S. griseus*	(Szeszak *et al.*, 1990, Birko *et al.*, 1999, Biro *et al.*, 2000, Birko *et al.*, 2007)
sigQ/ SCO4908	Putative sigma factor; disruption enhances antibiotic production and delays sporulation in *S. coelicolor*	(Shu *et al.*, 2009)
afsQ1/2/ SCO4907/6	Two-component system; stimulates antibiotic production in *S. lividans*; conditionally required for normal levels of antibiotic production and morphological development in *S. coelicolor*; regulates expression of *sigQ*	(Shu et al., 2009, Ishizuka *et al.*, 1992)

Orphan regulators and cluster-situated regulators (present at least in *S. coelicolor*)		
absB/ SCO5572	Endoribonuclease III, essential for antibiotic production in *S. coelicolor*; autoregulates own production; cleaves mRNA of *adpA*; disruption affects, but does not block sporulation process	(Price et al., 1999, Xu et al., 2010c, Xu et al., 2008)
absC/ SCO5405	Required for production of ACT and RED in *S. coelicolor* under conditions of limited zinc; directly represses gene cluster of coelibactin, a non-ribosomally synthesized peptide predicted to have siderophore-like activity; no obvious effect on morphological development	(Hesketh *et al.*, 2009)
absR1/2/ SCO6992/3	Essential for antibiotic production in *S. coelicolor*; no obvious effect on morphological development; located close to *absA*	(UHNMEE *et al.*, 2000)
cdgA/ SCO2817	diguanylate cyclase; enhanced expression blocks development and ACT production in *S. coelicolor*; regulated directly by BldD	(den Hengst *et al.*, 2010)
cutR/S/ SCO5862/3	TCS reported to repress ACT production in *S. lividans*	(Chang, 1996)
earE1/2/ SCO6421/2	Two-component system in *S. coelicolor*; located close to red cluster; disruption reduces level of RED produced	(Wang, 2007)
eshA/ SCO7699	Required for antibiotic production in *S. coelicolor* and normal development in *S. griseus*; accentuates (p)ppGpp accumulation; possible nucleotide-binding protein	(Wang, 2007)
nsdA/ SCO5582	Negative regulator of antibiotic production and development in several streptomycetes; target of BldD regulation	(den Hengst et al., 2010, Li, 2006, Wang, 2009)
nsdB/ SCO7252	Negative regulator of antibiotic production and development in several streptomycetes; transcribed divergently from gene of an Aph-like antibiotic phosphotransferase protein; contains a tetratricopeptide repeat domain	(Zhang L, 2007)
rapA1/2/ SCO5403/4	TCS; disruption reduces production of ACT and cryptic polyketide in *S. coelicolor*; appears to function via the corresponding URAPs; transduced signal is unknown	(Lu, 2007)
rrdA/ SCO1104	TetR family member; negatively regulates expression of *redD* (not yet known whether direct or indirect); impacts ACT production perhaps as a result of sharing precursors with RED	(Ou, 2009)
ssgA/ SCO3926	Cell division activator with pleiotropic effect on development; strongly enhances RED production and blocks production of ACT	(van Wezel *et al.*, 2000)
wblA/ SCO3579	WhiB-like transcription factor; disruption strongly increases antibiotic production in *S. coelicolor*; probably functions similarly in most, if not all, streptomycetes	(Kang *et al.*, 2007)

SCO1712	Transcriptional regulator that modulates antibiotic production in *S. coelicolor*; member of TetR family	(Traag & van Wezel, 2008)
cdaR/ SCO3217	CSR of the calcium-dependent antibiotic (cda) cluster	(Ryding et al., 2002)
mmyB/ CAC36754	URAP (transcriptional activator) of the methylenomycin (Mm) cluster on the SCP1 plasmid	(O'Rourke *et al.*, 2009)
mmfR/ CAC36768	Member of ArpA family; receptor for novel furans (related to gamma-butyrolactones) that autoregulate Mm production; functions in concert with MmyR; represses Mm and furan production until autoregulator accumulates	(O'Rourke et al., 2009, Corre *et al.*, 2008)

Other regulators have been unraveled their cellular functions and regulatory networks. NdgR (Yang et al., 2009) is known as a nitrogen source dependent growth regulator, Bld and Whi regulators and SsgA/SsgB (Traag & van Wezel, 2008) is related with morphological development. Quorum sensing regulatory networks are also revealed; AfsA related with A-factor synthesis in *S. griseus* (Kato et al., 2007), ScbR/ScbA as the gamma-butyrolactone regulatory system in *S. coelicolor* (Takano et al., 2001).

Still, people keep revealing the hidden layers of transcriptional regulatory network using various '-omics' tools. In this study, we also tried to understand the transcriptional regulatory networks using transcriptomics approaches. Piling up the more experimental data, the solider transcriptional regulatory networks would be constructed.

1.1.2 Functional study of transcriptional regulators in *Streptomyces*

Transcriptional regulators related to various cellular events have been also studied and they control multiple targets by DNA-binding (Hesketh et al., 2009), phosphorylation (McKenzie & Nodwell, 2007), RNA cleavage (Xu et al., 2010c) and transcriptional controls (Santos-Beneit et al., 2009, Santos-Beneit *et al.*, 2011). In the past, the transcriptional regulator study was performed by mutagenesis of target genes and people were observed several physiological changes followed by the mutation. As the technology has been developed, many analysis tools have been applied for functional study. One of the most valuable tools is microarray, which will be mentioned in section 1.2.1. DNA microarray. Moreover, other methodologies for high-throughput screening are also developed.

According to our previous work, DACA (DNA affinity capture assay) is a powerful method to find a novel transcriptional regulator which is bound to specific DNAs (Park et al., 2009). By DACA, we could know the target regulators which bind the known promoter region and it is possible to get the clues about the transcriptional regulators to understand the transcriptional networks. For example, from DACA experiments, NdgR is screened and characterized as a nitrogen-source dependent growth regulator, which affects not only antibiotics production but also morphological changes (Yang et al., 2009). In addition, media selection using 96 well plates containing minimal media with different carbon sources and nitrogen sources is an efficient method for primary screening of physiological changes between wild type and mutants and this makes the characterization of transcriptional regulators easier (Yang et al., 2009). Measurement of intracellular intermediate molecules and pH changes also gives hints of the functions of target regulators (Yang *et al.*, 2010). Because all the observed changes occur by mutation of regulator.

1.1.3 Strategies to enhance antibiotic production

Most pharmaceutically useful secondary metabolites such as antibiotics are produced by streptomycetes. In spite of the importance of secondary metabolites, it is still not easy to overproduce chemically because they need many modification steps by which their complex structures are made. Therefore, many studies were preceded to use streptomyces as host strains. Various approaches for enhancing secondary metabolites were studied, and one of them is to increase the pool of cofactors or precursors in host cells. For example, CoA derivatives are the precursors of many antibiotics and

useful fatty acids. Therefore, increasing the CoA pool in host cell is the fundamental procedure (Guilfoile & Hutchinson, 1991, Murakami *et al.*, 2011, Tahlan *et al.*, 2007). The other approach is to insert important genes or gene clusters related with the secondary metabolites biosynthesis into host cell by genetic engineering. Recently, many antibiotics were made by the heterologous expression (Park *et al.*, 2011, Qiu *et al.*, 2011) and by the insertion of the multi-copy antibiotics biosynthetic gene cluster (Murakami et al., 2011, Yanai *et al.*, 2006). Other approaches are the substitution of the specific promoter (Blount *et al.*, 2012, Guo *et al.*, 2012, Jensen & Hammer, 1998), to control the unwanted pathway (Kurumbang *et al.*, 2010, Li & Townsend, 2006), to promote the resistance of cytotoxicity by the over-expression of antibiotics resistant genes or efflux pumps (Dairi *et al.*, 1995, Malla *et al.*, 2010, Yanai et al., 2006), the engineering of the global or pathway-specific regulators (Adamidis & Champness, 1992, Bruheim *et al.*, 2002, McKenzie & Nodwell, 2007, Takano *et al.*, 1992, Uguru et al., 2005) and so on.

In *streptomyces*, there are complicated transcriptional regulatory network in order to control many cellular events, especially the secondary metabolism (Martin & Liras, 2010). Transcriptional regulators modulate the expression of target genes by binding the specific sites of DNA. Moreover, transcriptional regulators target several genes which are related with particular function, it means that it is possible to affect several genes at once by over-expression or deletion of transcriptional regulators. Therefore, the engineering of transcriptional regulators is effective approach to overproduce secondary metabolites which have complex network.

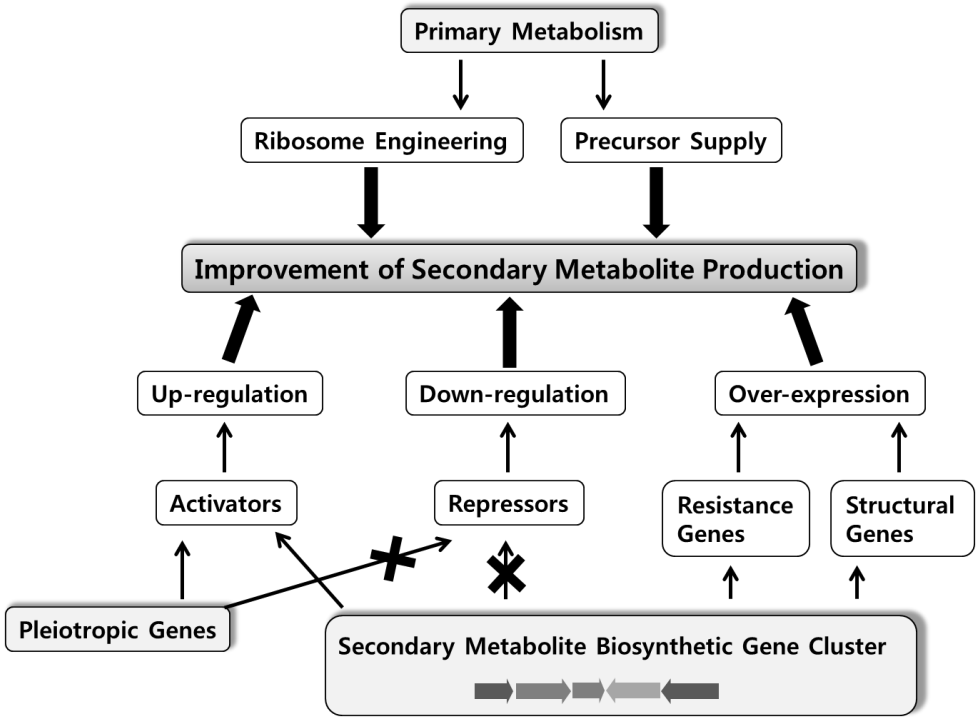


Figure 1.1 Various approaches to produce secondary metabolites. Modified from (Olano *et al.*, 2008)

1.2 Transcriptomics based approach to study TR network

From the first nucleotide sequence analysis, genomes of many organisms have been sequenced and people could obtain the whole genomic information. Over the decades, sequencing techniques have been developed and genome sequencing can be performed easier and the amount of data becomes larger. By the sequencing analysis, the more information about many organisms are available, however, there were still limits to study the functions of the specific genes using the genome data. Therefore, several '-omics' approaches have been applied for understanding the complicated cellular biology. For example, transcriptomics is the study of using mRNA transcript levels. Proteomics is the study which measures the protein abundance, metabolomics is the study about quantifying the amounts of cellular metabolites, interactomics is the study of analyzing the molecular interactions in the cells, and fluxomics is the study which analyzes the dynamics of cellular molecules over time (Zhang *et al.*, 2010, Fiehn, 2001, Kandpal *et al.*, 2009, Lin & Qian, 2007, Singh & Nagaraj, 2006, Tang *et al.*, 2009). '-Omics' approaches are considered that it is high-throughput and top-down based method compared to traditional analysis methods. Moreover, various statistical analysis methods are needed to interpret the '-omics' data since we could obtain the huge data sets. Combining the '-omics' tools to analyze transcripts, proteins and metabolites, we could explain the cell biology (Joyce & Palsson, 2006, Steinfath *et al.*, 2007).

Transcriptomics is a kind of functional genomics. It means that we could assume the cellular functions of the specific gene by the mRNA expression levels. Transcripts in one cell or a cell community are globally changed when the extracellular signals such as the nutrient starvation,

quorum signaling and the mutation of the target genes are given. Therefore, transcriptomic approach is suitable to observe the changes of mRNA expression patterns occurred by the target transcriptional regulators and to estimate the cellular functions of the target transcriptional regulators from those changes. In this study, we could use transcriptomic data from microarrays to infer the transcriptional regulatory network in *Streptomyces coelicolor*. We suppose that the complete transcriptional regulatory networks are described soon as the transcriptomic data are accumulated more.

1.2.1 DNA microarray

Traditional methods for unraveling the functions of the genes are quite limited. The available range of observation and the obtained data were small since it used gene-by-gene approach. However, the techniques have been developed, and we could choose proper tools for studying the functions of genes.

Recently, the common tools for transcriptomics are microarrays (Schena *et al.*, 1995, Lockhart *et al.*, 1996), serial analysis of gene expression (SAGE) (Velculescu *et al.*, 1995) and next generation sequencing (NGS) such as RNA-Seq (Marioni *et al.*, 2008, Wang *et al.*, 2009, Cho *et al.*, 2009) and ChIP-Seq (Mardis, 2007). All analysis tools are correlated with the population of RNA transcripts in the cells.

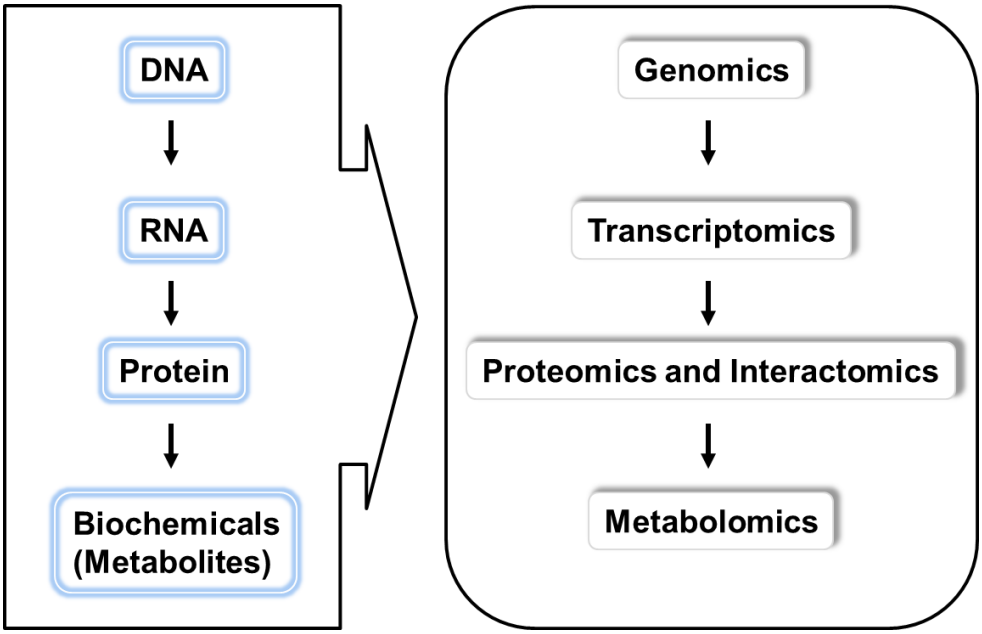


Figure 1.2 Various '-omics' approaches targeting different molecules. Modified from (Zhang et al., 2010).

The basic procedure of microarray experiment is that RNA transcripts from the samples are changed to cDNA by reverse transcription polymerase chain reaction (RT-PCR) and labeled with fluorescent dyes. Then, dye-labeled cDNA are hybridized with the probes immobilized in the solid surfaces of microarray. Therefore, we can detect the fluorescent signals by scanning the microarrays and we assume the cellular function of target genes by analyzing the global changes of transcripts abundance between samples. The fundamental assumption of microarray experiment is that the hybridization between cDNAs and probes would be performed theoretically in the same manner, and from that assumption, we can analyze the measurements of mRNA expression levels. However, it is needed to normalize the microarray data since there are possible to have systematic and experimental errors by characteristics of fluorescent dyes or different labeling efficiency in the dual dye-labeling system and hybridization efficiency according to the probe design.

In spite of some drawbacks, those can be overcome during data analyzing process. Microarray experiments are simple and become cheaper than before, therefore it is clear that the frequency of use would be increased.

1.2.2 Next generation sequencing

Hybridization based methods like microarrays have several disadvantages mentioned in section 1.2.1 DNA microarray. Therefore, sequencing based methods rather than hybridization based methods attract the attention. Those methods are SAGE and next generation sequencing (NGS) such as RNA-Seq and ChIP-Seq. In contrast with the SAGE which uses unique tag

to each gene for sequencing, RNA-Seq uses adaptors to ligate the RNA transcripts. Then, adaptor-ligated RNA transcripts were converted to cDNA and directly sequenced in a high-throughput way by Illumina IG, Applied Biosystems SOLiD and Roche 454 Life Science systems (Metzker, 2010).

Interestingly, RNA-Seq has several advantages compared to the microarray system. First advantage is that RNA-Seq has low background signals because of direct sequencing from RNA samples. Therefore it is no need to perform amplification steps and additionally from that point, the quantification of RNA transcript abundance becomes more accurate. Second advantage is that there is not necessary to design the probes for hybridization, therefore it is possible to use RNA-Seq for analyzing the transcriptome of un-sequenced organisms. Moreover, it can be obtained the information of small RNAs which is not located in the ORF regions.

In addition, ChIP-Seq is the method that immuno-precipitated nucleotides directly sequenced like RNA-Seq. According to ChIP-Seq method, we can infer *in vivo* transcriptional regulatory network.

To understand the transcriptional regulators and regulatory networks, the comprehension of whole transcriptomes in the cells are quite important. Therefore, transcriptomics approaches like RNA-Seq, ChIP-Seq and microarrays as well as integrating '-omics' approaches become more significant.

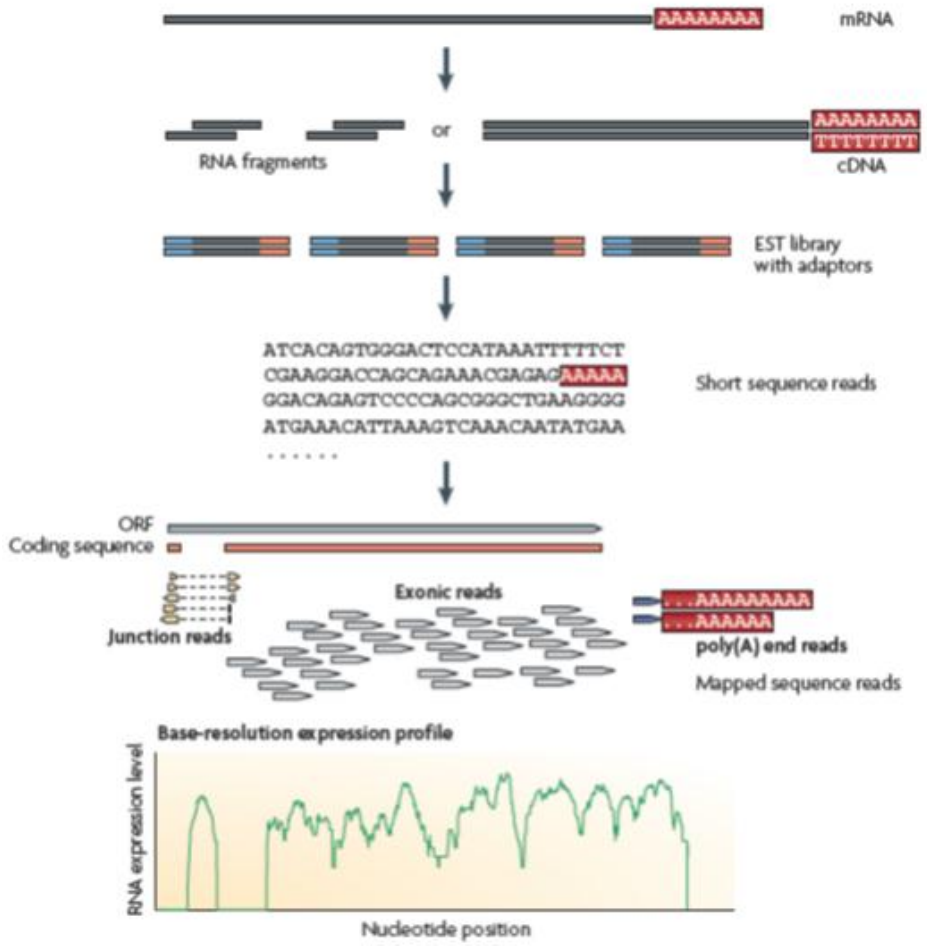


Figure 1.3 The procedure of the RNA-Seq experiment. This is the case of eukaryotic samples. In bacteria, it cannot be possible to use poly(A)-tail. Adopted from (Wang et al., 2009)

1.3 The scope of thesis

In Chapter III, it is discussed about the analysis of novel interacting transcriptional regulators related to antibiotic production using microarray. The role of AbsB is known as the higher level regulator and is controlling the antibiotic production as an activator and morphological development. We focused the cellular function of AbsB and selected several candidates to be regulated by AbsB depending on their changes of mRNA expression pattern using time-course microarray data. By *in vitro* ribonucleolytic cleavage assay, we could observe that AbsB controlled the lower level regulator, SCO6808. Therefore, we could identify the novel interaction between regulators in the multi-layered transcriptional network.

Then we did not remain the stage to interpret the transcriptional regulatory network, we attempted the application of transcriptional regulatory network for overproducing antibiotics. On the side of antibiotics over-production, the over-expression of activators or the deletion of repressor would be good approaches for controlling transcriptional regulatory networks. However, we supposed to maximize the antibiotics production by using the combination of regulators not a single regulator. In Chapter V, we tried to find the target regulators that were effective to make the combination with *afsS* using microarray data of *afsS* disruption mutants and to make the double mutants with the combination of AfsS and transcriptionally independent regulators, SCO4677 and PhoU. Therefore we were possible to dramatically increase antibiotics using the double mutants.

We still needed more time-course transcriptomic information to figure out the regulatory network in *Streptomyces coelicolor*. Therefore, time-course DNA microarray experiment was introduced in Chapter IV.

NdgR was selected the target regulator. NdgR was reported as a global regulator that controls amino acids synthesis, quorum sensing and antibiotics production in previous report. In addition to those roles of NdgR, we newly revealed that NdgR also controls amino acids transport system, for example up-regulation of glutamate and branched amino acid transport system and down-regulation of methionine transport system, and carbohydrate uptakes and carbohydrate metabolism such as down-regulation of PTS and up-regulation of glycerol metabolism, and cell stress response and tolerance to toxic materials. Those finding were confirmed by EMSA, cell viability test, the observation by confocal microscope and TEM images. By time coursed microarray experiment, we could reveal the new cellular functions of NdgR and accumulate the information about the role of the global regulator, NdgR to the incomplete transcriptional regulatory network in *Streptomyces coelicolor*.

Still, multi-layered complex transcriptional regulatory network is unclear and small proportion of transcriptional regulators unraveled their cellular roles and hierarchical interactions in the network. Addition to transcriptomics and proteomics approaches such as microarray, 2D-gel electrophoresis and mass spectrometry, next generation sequencing techniques such as RNA-seq, methylation-seq and ChIP-seq have newly developed. Therefore, it would be possible to pile up more precise and massive information about transcriptional regulators. Especially, we would be obtained the *in vivo* information about transcriptional regulator by ChIP-seq. More data of DNA chip datasets, RNA-seq and ChIP-seq are needed to integrate to our regulatory network and it will be possible to lead us the way of understanding *Streptomyces*.

Chapter II

Materials and Methods

2.1 Bacterial strains and plasmids

Streptomyces coelicolor M145 obtained from KCTC (Korean Culture and Tissue Collection) were cultured and maintained as described by Kieser *et al*. Strains used in this study were listed in Table 2.1.

2.2 Growth media and culture conditions

Cultivation of *S. coelicolor* strains followed the standard procedures. In liquid culture, about $10^{6\sim7}$ spores/μL spores were inoculated and cultured for 20 hrs in $R5^{-}$ media first. $R5^{-}$ media were composed of 103 g sucrose, 0.25 g K_2SO_4, 10.12 g $MgCl_2•6H_2O$, 10 g glucose, 0.1 g Difco casamino acids, 2 ml of a trace element solution composed of 40 mg $ZnCl_2$, 200 mg $FeCl_3•6H_2O$, 10 mg $CuCl_2•2H_2O$, 10 mg $MnCl_2•4H_2O$, 10 mg $Na_2B_4O_7•10H_2O$, 10 mg $(NH_4)_6Mo_7O_{24}•4H_2O$ in 1 L of distilled water, 5 g yeast extract, 5.73 g TES buffer, and 7 ml of 1N NaOH in 1 L of distilled water. The germinated cells were harvested by centrifugation at 13,000 rpm and washed with sterilized distilled water three times before inoculation (0.1 g/mL wet weight) into 25 mL or 50 mL of new media.

Supplemented minimal media (SMM) contained 2 g Difco casamino acids, 5.73 g TES buffer in 1 L of distilled water were adjusted to pH 7.2 with 5 N NaOH, and added final 5 mM $MgSO_4$ (1 M), final 50 mM glucose (50% w/v), 1 ml of a trace element solution composed of 0.1 g $ZnSO_4•7H_2O$, 0.1 g $FeSO_4•7H_2O$, 0.1 g $MnCl_2•4H_2O$, 0.1 g $CaCl_2•6H_2O$, 0.1 g NaCl in 1 L of distilled water, and without or with various final concentration of 50 mM each of NaH_2PO_4 and K_2HPO_4. Solid SMM and liquid SMM had same compositions except agars, which were 15 g in 1 L solid SMM.

Table 2.1 Strains, plasmids, and primers used in this study

Strain, plasmid, and primer	Relevant information
Bacterial strains	
E. coli	
DH5α	F$^-$ φ80*lacZ* M15 *endA recA hsdR*($r_k^-m_k^-$) *supE thi gyrA relA* Δ(*lacZYA-argF*)U169
BL21(DE3)	F$^-$ *ompT hsdS$_B$*($r_B^-m_B^-$) *gal dcm*
JM110	*dam*$^-$, *dcm*$^-$
BW25113	K12 derivative: Δ*araBAD*, Δ*rhaBAD*
ET12567	*E. coli* methylation-deficient strain (*dam*$^-$, *dcm*$^-$, *hsdM*$^-$) used for conjugation between *E. coli* and *Streptomyces*
S. coelicolor	
A3(2) M145	SCP1$^-$, SCP2$^-$,Pgl$^+$
Δ*sco4677*	*sco4677* deleted mutant
Δ*phoU*	*sco4228* deleted mutant
M145+ pIBR25::*phoU*	M145 containing pIBR25::*sco4228*
M145+ pIBR25::*afsS*	M145 containing pIBR25::*sco4425*
BG11	*sco5552* (*ndgR*) deleted mutant
BG4677S	*sco4677* deleted mutant containing pIBR25::*afsS*
BG4228S	*sco4228* deleted mutant containing pIBR25::*afsS*
BG4677	*sco4677* deleted mutant containing pIBR25
BG4228	*sco4228* deleted mutant containing pIBR25
Plasmids	
pET28a	T7 lac promoter, N-and C-terminal his-tag coding, kan^R
pSET152	*lacZα ori*pUC19 *oriT*RP4 *int-attP*φC31 *aac*(*3*)*IV*

pKC1139	Temperature-sensitive *E. coli - S. coelicolor* shuttle vector
pIJ773	*aac(3)IV*(Apra^R)+*oriT*
pSuperCos1	A cosmid vector that contains bacteriophage promoter sequences
pWHM3	Multicopy *Streptomyces* and *E. coli* shuttle vector, tsr^R
pIBR25	pWHM3 carrying *ermE** promoter (*Eco*RI/ *Kpn*I) from *Saccharopolyspora erythraea*
pIBR25::*afsS*	pIBR25 carrying PCR product of *afsS* from *S. coelicolor*
Primers	
Δ4677-F	5’-ttctcacggtacgcacacatcgccacgctgagtgacgtgattccggggatccgtcga cc-3'
Δ4677-R	5’-ttcttgtcttggtagggcgccgacggcaccggaacctcatgtaggctggagctgcttc-3’
Δ4228-ecoF1	5’- atatgaattcgagagcttcttcgccgagtgg -3’
Δ4228-xbaR1	5’- atatattctagacatcaggattccctctcgtac -3’
Δ4228-ecospeF2	5’- atatgaattctatatactagtcaggtcgagggagcctgagc -3’
Δ4228-xbaR2	5’- atatattctagacagccgagctgcggttcgag -3’
::4228-BamF	5’- atatggatccttcatttggggtgccggaaac -3’
::4228-HindR	5’- atataagctttcaggctccctcgacctgcgt -3’
::AfsS-XbaF	5’- ttaatctagacaccagttagtgtgcggctc-3’
::AfsS-EcoR	5’- atatgaattcctacttgccgtcgccgtccagg
AbsB-His$_6$-F	5’- TGAGAGGATCCATGTCAGTCCCCAAGAAGGC
AbsB-His$_6$-R	5’-TGGACAAGCTTGGCGGAGGCGGACGCCTCGT
sco6808-F	5'-**TAATACGACTCACTATAGGG**AGAAAGCCATAGCCAACAAGGACCG
sco6808-R	5’-AGATGGAGGGAGATCCGGAA
sco1839-F	5'-**TAATACGACTCACTATAGGG**TCGCCCTCGCTTGATCGAATGGATG
sco1839-R	5’-TGGCCACCGTCACTTTCGGA

EMSA-gluA	TTGCGGCCGCTCGAAGTTAT/TACTTCGGTCATCGCTCTCG
EMSA-glycerol	AGAGTGTCCACAGTGCCAAG/
	AGATGTCGGAGCTGGACACC
EMSA-PTS	GTTCACAGCGTCCTCGCTGT/
	CCAAGAGAGTCTCTTGGGTG

Note: The underlined letters indicate BamHI and HindIII sites; the bold letters indicate the bacteriophage T7 promoter.

Minimal media were composed of 0.5 g L-asparagine as a nitrogen source, 0.5 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 10 g N-acetyl glucosamine as a carbon source in 1 L distilled water. Solid MM and liquid MM had same compositions except agars, which were 22 g in 1 L solid MM.

E. coli was cultivated at 37℃ in LB (0.5% yeast extract, 1% tryptone, 1% NaCl) supplemented with appropriate antibiotics.

Cells were cultivated vigorously in 100 mL or 250 mL Erlenmeyer flasks with an orbital shaking incubator (200 rpm) and glass beads at 30℃. The cell growth was measured by wet cell weight or OD_{450nm} for *S. coelicolor*.

E. coli DH5α and *S. coelicolor* M145 protoplast cells were used as a host for plasmid constructions. *E. coli* ET12567/pUZ8002 , which supply the donor function (trans-) when having a compatible *ori*-T-containing plasmid, was used to get non-methylated DNA for intergeneric conjugation with *S. coelicolor* M145. *E. coli* BL21 (DE3) was used to over-express recombinant proteins based on T7 promoter system according to the manufacturer's recommendations (Novagen).

2.3 DNA manipulations

2.3.1 Plasmids and recombinant preparation

pIBR25 containing *ermE** promoter which is a high-copy number shuttle vector was used as cloning vector for preparing a recombinant DNA and then transformed into *S. coelicolor* for over-expression of the recombinant DNA. The chromosomal DNA of *S. coelicolor* M145 was prepared with

genomic DNA extraction kit as described by the manufacturer's recommendations (iNtRON Biotechnology, INC). For the over-expression of the target genes, they were amplified with its own forward primer and backward primer described in Table 2.1.

2.3.2 Construction of knock-out mutants – PCR targeting

PCR amplification of the extended resistance cassette

A target gene to be deleted was deleted by PCR targeting (Kieser *et al.*, 2000) through the replacement of the entire coding region by an apramycin resistance cassette (Figure 2.1). For each gene disruption, two long PCR primers (58 nt and 59 nt) were required. Each had at the 5´ end 39 nt matching the *S. coelicolor* sequence adjacent to the gene to be inactivated, and a 3´ sequence (19 nt or 20 nt) matching the right or left end of the disruption cassette (all cassettes have the same "right" and "left" ends). All PCR amplifications were performed using the Expand high fidelity PCR system in a total 50 μl reaction volume with each 50 pmole primer, 100 ng template DNA, 5% DMSO, each 50 μM dNTP, 2.5 Unit Pfu polymerase, and 5 μl 10x buffer according to the manufacturer's instructions (Roche). Cycle conditions were as follows: denaturation at 94℃, 2 min, then 10 cycles with denaturation at 94℃, 45 sec, primer annealing at 50℃, 45 sec, and extension at 72℃, 90 sec, and then other 15 cycles with denaturation at 94℃, 45 sec, primer annealing at 55℃, 45 sec, extension at 72℃, 90 sec, and final extension at 72℃, 5 min.

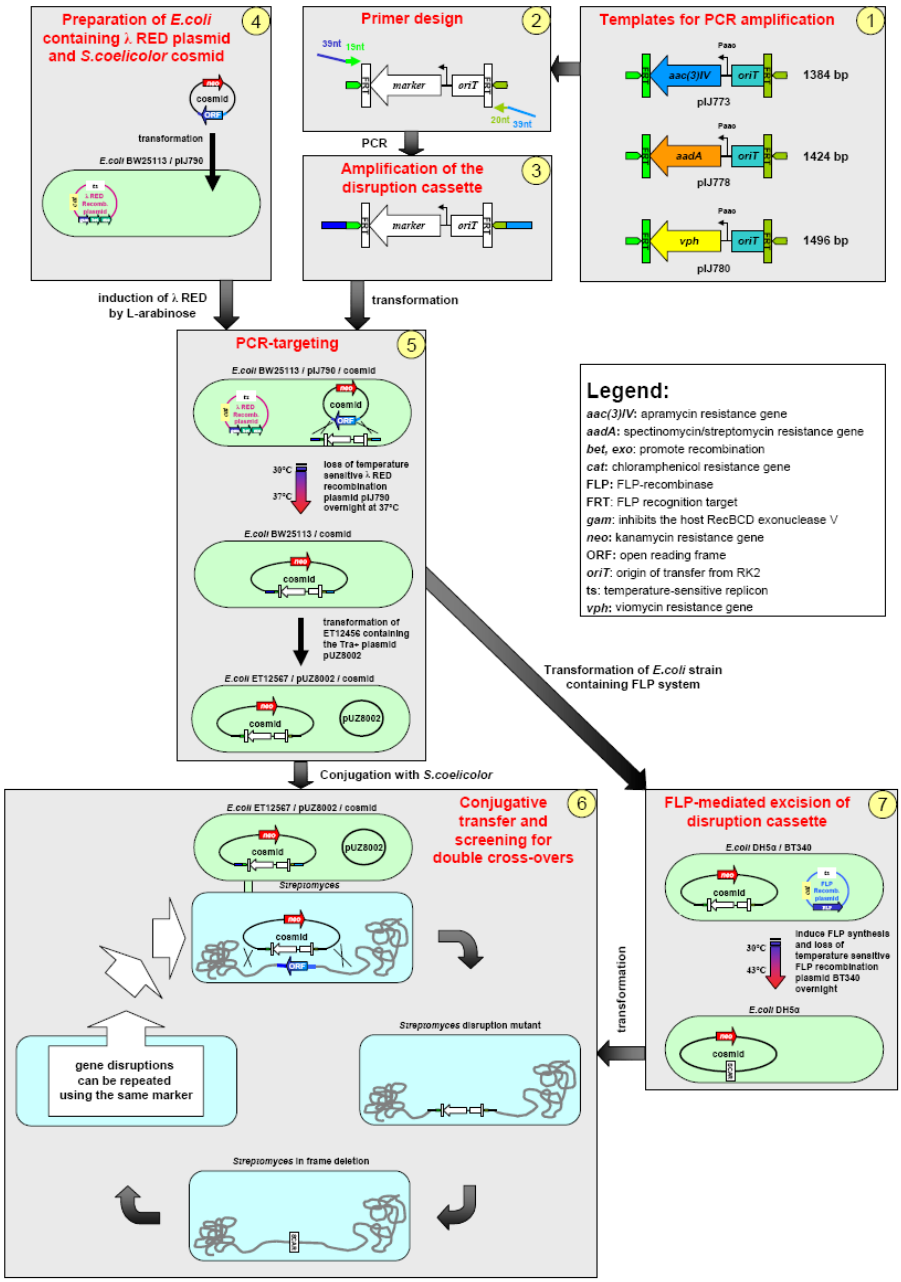


Figure 2.1 Flow chart of gene disruption by PCR targeting. Adopted from (Gust *et al.*, 2003).

PCR targeting from the *S. coelicolor* cosmid

Electro-competent *E. coli* BW25113/pIJ790 and the *S. coelicolor* cosmids which containing the target genes to be deleted were cultured in a 10 mL SOB containing carbenicillin (100 μg/mL), kanamycin (50 μg/mL), chloramphenicol (25 μg/mL), and 10 mM L-arabinose which induces *red* genes. After reaching to an OD_{600} of ~ 0.6, the cells were centrifuged at 3,800 x g for 5 min at 4℃ and washed with ice-cold 10% glycerol twice. And then the cell pellet resuspended in remaining ~ 100 μL 10 % glycerol. 50 μl electro-competent cell suspension electroporated with ~ 100 ng of PCR product in a 0.2 cm ice-cold electroporation cuvette using a BioRad GenePulser II set to: 200 Ω, 25 μF and 2.5 kV. The expected time constant was 4.5 – 4.9 ms. Shocked cells incubated shaking 1 h at 37℃ in 1 mL LB and then spreaded onto LB agar containing carbenicillin (100 μg/mL), kanamycin (50 μg/mL) and apramycin (50 μg/mL). The gene disruption was confirmed by PCR.

The complete coding region of *sco4677* was deleted from the corresponding cosmid (StD31) by PCR targeting using oligonucleotide primers (Table 2.1) with 5' ends overlapping the upstream (36bp) and downstream (36bp) of the *sco4677* coding sequence and 3' (priming) ends containing start and stop codons. These primers were designed to amplify the pIJ773 cassette containing the apramycin resistance gene (Table 2.1). The deletion of *sco4677* was confirmed by sequencing of corresponding PCR products.

2.3.3 Construction of knock-out mutants

Disruption of *sco4228* (*phoU*) was performed using a plasmid pKC1139 to deliver the kanamycin resistance gene cassettes (Table 2.1). The cassette consisted of two PCR-derived flanking regions (1kb) in which suitable restriction sites were introduced. Disruption of *sco4228* (*phoU*) was performed using a plasmid pSuperCos1 to deliver the corresponding apramycin resistance gene cassettes (Table 2.1). The cassette consisted of two PCR-derived flanking regions (2.7kb) in which suitable restriction sites were introduced. Detailed primer information for each construction is summarized in Table 2.1. All of the constructs were delivered into *S. coelicolor* M145 by conjugation with *E. coli* ET12567. Conjugation between *E. coli* and *Streptomyces* was performed as described previously. The transformants were selected on R2YE/kanamycin medium for *sco4677* deletion and R2YE/apramycin medium for *sco4228* (*phoU*) deletion, and then confirmed by PCR.

2.4 Electrophoretic mobility shift assay (EMSA)

Each promoter was amplified by PCR with fluorescent FAM labeled 5' primers and purified using ProbeQuant^{TM} G-50 Micro columns (GE Healthcare, USA). The labeled probes were incubated with His-tag purified proteins, AbsB-His_6 for 30 min at room temperature. The total volume is 20 µL containing 20 mM TBE (Tris/ Borate/ EDTA) buffer, 10% w/v glycerol, 100 mM KCl, 0.05 mM EDTA, 5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), and 2 µg sheared salmon sperm DNA (sssDNA). Protein-bound DNA and free DNA were resolved on 5% acrylamide gels in 0.5X TBE buffer at room temperature. The gels were analyzed with Typhoon 8600 scanner

(Molecular Dynamics).

2.5 Semiquantitative RT-PCR

RNA obtained as described in materials and method section 2.6 was reverse transcribed into first-strand cDNA in the presence of 1 μg total RNA, 5x First-Strand Buffer, 0.5 mM dNTP mix, 200 unit SuperScript^{TM} III Reverse Transcriptase (Invitrogen, USA), 40 unit RNaseOUT^{TM} recombinant RNase inhibitor, 3 μg of random hexamer, 5 mM DTT in a final reaction volume of 20 μL according to the manufacturer's instructions. Reactions were carried out in a water bath at 25℃ for 5 min, at 50℃ for 30 min incubation, followed by heating to 70℃ for 15 min to denature the enzyme, and then cooling to 4℃. The synthesized cDNA was amplified by PCR in a total 50 μl volume with each 10 pmole primer, 100 ng cDNA, 1 unit LA Taq DNA polymerase (TAKARA), 2X GC buffer, each 200 μM dNTP. 25 cycles of amplification were carried out where it was ensured that DNA amplification was in the linear range for each template on kinetic analysis. 16S rRNA was used as a control to normalize the expression level of each mRNA and sample-to-sample variations. The PCR products were visualized with the use of 2% w/v agarose gels stained by ethidium bromide.

2.6 DNA microarray

Culture conditions

Fresh spores of *Streptomyces coelicolor* M145 and BG11 were collected on R5^{-}. Spores of M145 and BG11 were cultured in two 100 mL flask containing 25 mL R5^{-} complex liquid media for 15 h and the mycelia were

harvested by centrifugation at 13,000 rpm and washed for three times with sterilized water. Same amount of M145 and BG11 were inoculated on the cellophane covered surface of solid minimal media containing 10 g N-acetyl glucosamine as a carbon source and 0.5 g L-asparagine as a nitrogen source, 0.5 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 22 g agar and 0.01 g $FeSO_4 \cdot 7H_2O$ in 1 L distilled water.

Total RNA extraction

Cells were scrapped from cellophane membranes of solid minimal media at 36, 48, 60 and 72 h and immediately treated with RNAlater (Sigma, USA) for preventing RNA degradation. Then samples were frozen and ground under liquid nitrogen using a mortar and pestle. Cell powders were moved into an RNase-free 50 ml falcon tube and immediately mixed with the RLT solution included in the RNeasy mini kit (Qiagen, USA). RNA extractions were performed following with the manufacturer's instructions of the RNeasy mini kit (Qiagen, USA).

Using a NanoDrop ND-1000 spectrophotometer, the concentration and the purity of RNA were determined relying on the absorbance at 260 nm (A260) and the ratio of the absorbance at 260 nm and 280 nm (A260/A280) and the ratio of the absorbance at 260 nm and 230 nm (A260/A230). Pure RNA has the A260/A280 ratio of 1.8-2.1 and the A260/A230 ratio of 1.8-2.2. If contaminants such as proteins, phenol, carbohydrates and other organic acids remain in the RNA samples, the ratio of A260/A280 and the ratio of A260/A230 values indicate below 1.8.

Moreover, the integrity of RNA samples was checked by microfluidics technology using Agilent 2100 Bioanalyzer using 1 μL of

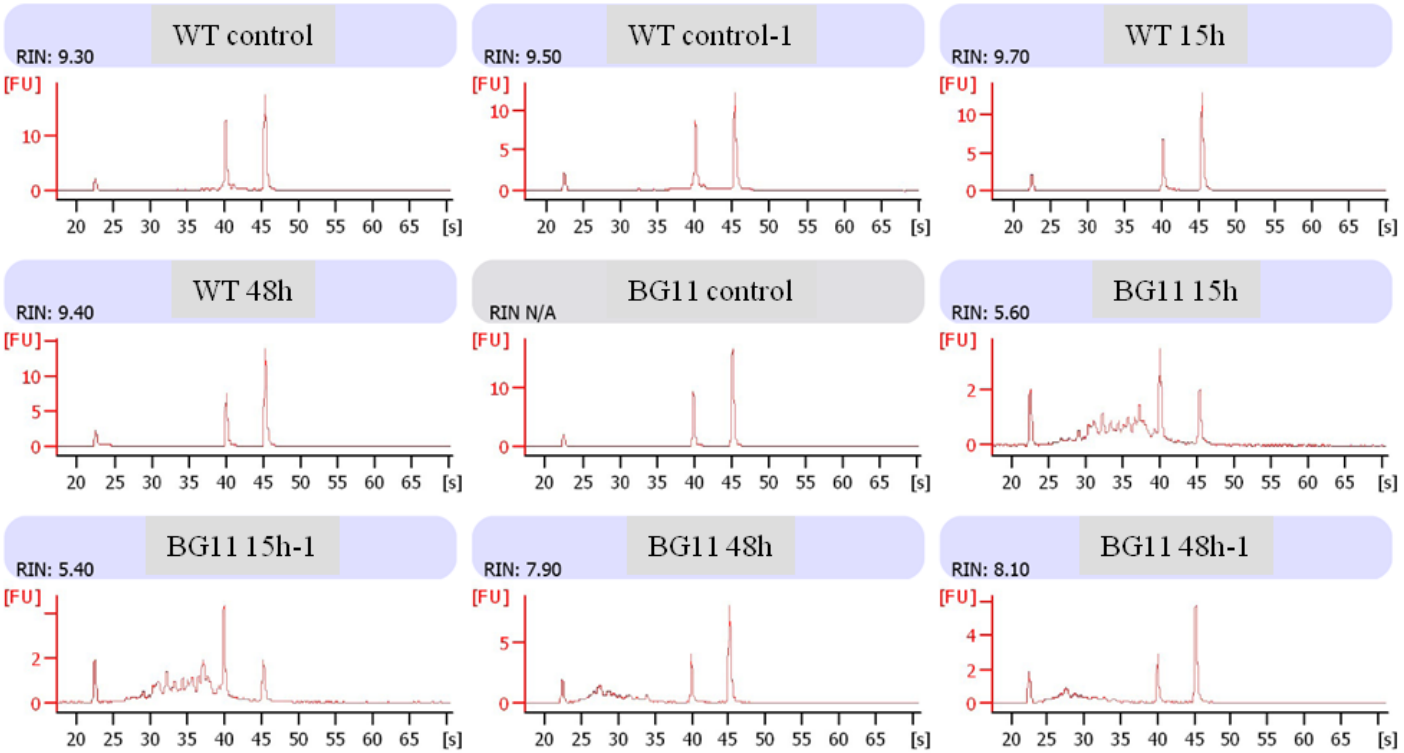
RNA. The integrity check is important step because the RNA stability is determined by the value of RNA integrity which is the ratio of 23S ribosomal RNA (rRNA) and 16S rRNA. Therefore, the ratio of 23S/16S rRNA of non-degradaded RNA is more than 1.8. In Agilent 2100 Bioanalyzer, RNA stability can also be acquired by RIN (RNA integrity number) and RIN should be better above 8.0.

All RNA samples used in the study had above 1.8 ratio of A260/A280, A260/A230, 23S/16S rRNA and more than 7.5 of RIN.

cDNA synthesis and labeling procedure

All procedures followed with the manufacturer's instructions of the FairPlay III Microarray Labeling kit. For cDNA synthesis, 8-10 μg of total RNA was diluted to 12 μL with nuclease-free water and mixed with 1 μL of 500 ng/μL random primer gently. The solution was incubated at 70 °C for 10 min and cooled on ice until ready for use in order to anneal primer and RNA. The annealed primer and RNA were combined 2 μL of 10X AffinityScript RT buffer, 1 μL of 20X dNTP mix with amino allyl dUTP, 1.5 μL of 0.1 M DTT and 0.5 μL of 40 U/μL RNase Block. Then, 3 μL of AffinityScript HC Reverse Transcriptase were added and the mixture was incubated at 42 °C for 60 min. To hydrolyze RNA, 10 μL of 1 M NaOH were added and the solution was incubated at 70 ℃ for 10 min and cooled to room temperature slowly. Then, 10 μL of 1 M HCl were added to neutralize the solution. For cDNA purification, unincorporated nucleotides, buffers, and hydrolyzed RNA were removed by ethanol precipitation.

(a)


WT control
RIN: 9.30
WT control-1
RIN: 9.50
WT 15h
RIN: 9.70
WT 48h
RIN: 9.40
BG11 control
RIN N/A
BG11 15h
RIN: 5.60
BG11 15h-1
RIN: 5.40
BG11 48h
RIN: 7.90
BG11 48h-1
RIN: 8.10
[FU]
[s]

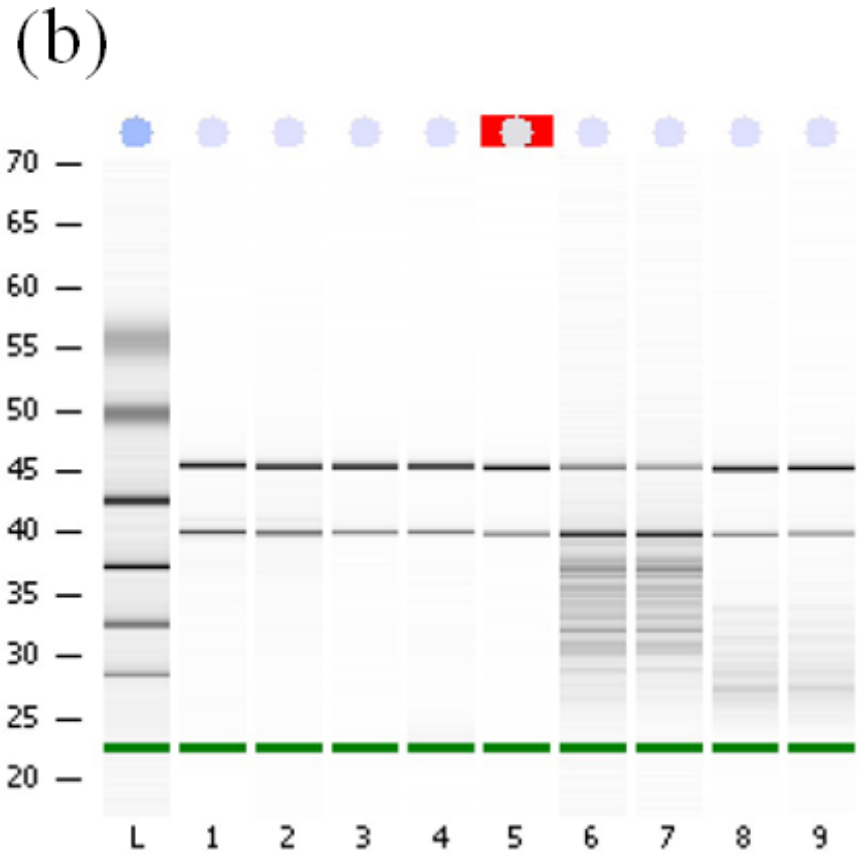


Figure 2.2 Examples of RNA quality checks using samples of wild type and BG11 for microarray experiments. Chromatograms of RNA samples (a), and gel images of the same RNA samples (b). Samples in lane 6 and lane 7 were degradaded.

For labeling the cDNA with CyDye^{TM} Cy3 mono-reactive dye (GE Healthcare), cDNA pallet were resuspended in 5 μL of 2X coupling buffer. It was added 5 μL of dye in DMSO to the cDNA and incubated for 30 min at room temperature in the dark place. The NHS-Ester conjugated dyes were evenly coupled with the reactive group of amino allyl modified cDNA. After that, buffer salts and uncoupled dyes were removed by the purification of ethanol precipitation.

Quantification of the fluorescent cDNA was performed by a NanoDrop ND-1000 spectrophotometer. Using 1 μL of fluorescent cDNA, concentration and labeling ratio were checked and the concentration of cDNA should be more than 13 ng/μL.

Hybridization and microarray slide scanning

600 ng of Cy3-labeled cDNA were mixed with 5 μL of 10X Blocking Agent and nuclease-free water was brought volume to 25 μL. Then, 25 μL of 2X HI-RPM GE Hybridization buffer were added to cDNA mixture and mixed gently by pipetting to avoid introducing bubbles.

For hybridization, a clean gasket slide was loaded into the Agilent SureHyb chamber and 40 μL of hybridization sample were slowly dispensed onto the gasket. Microarray slide was put the active side in which probes were immobilized down and paralleled to the SureHyb gasket slide. The SureHyb chamber cover was placed onto the slide and the gasket slide and firmly tightened. The slide chamber was placed in a hybridization oven and set the hybridization rotator to rotate at 10 rpm and hybridized at 65 ℃ for 17 h.

Before the microarray washing, 10 % Triton X-102 was added to

Gene Expression wash buffer 1 and Gene Expression wash buffer 2 until the final concentration of wash buffer 1 and 2 were reached 0.005 %. Gene Expression wash buffer 2 was pre-warmed to 37 ℃. After confirming the hybridization performed properly, the assembled slide glasses were placed into the first dish contained Gene Expression wash buffer 1 at room temperature and carefully removed the gasket slide from the array slide. The array slide was quickly moved to the slide rack in the second dish contained the Gene Expression wash buffer 1 with a magnetic stir bar at room temperature for preventing drying of the array slide and the slide was stirred for 1 min. The slide rack was transferred to third dish contained pre-warmed Gene Expression wash buffer 2 with a magnetic stir bar and was also stirred for 1 min. The slide rack was very slowly removed from the dish in order to minimize droplets on the slide. The slide was put in a slide holder and immediately scanned with Agilent High-Resolution Microarray Scanner (Agilent).

Fluorescent intensities were measured by the microarray scanner and after first scanning, setting the sensitivity by controlling the PMT value was performed for balancing between arrays. Total intensity values were organized using Microsoft EXCEL program for further data processing.

Microarray design

Whole genome data of *Streptomyces coelicolor* A3(2) were obtained from NCBI genome (ftp://ftp.ncbi.nlm.nih.gov/genomes/Bacteria/Streptomyces_coelicolor_A3_2__uid57801/NC_003888.ffn). On the Agilent website (https://earray.chem.agilent.com/earray/), genome data were submitted for probe design. Array design format was 8 x 60K and this meant that

maximum 60,000 probes were possible for one sample and 8 samples were in one array slide. One probe-set had two probes per one gene and triplicated probe-sets were fixed on one sample array. Location of the immobilized probes were randomized not a gene numerical order.

2.7 DNA array data analysis

To validating the obtained microarray data for further analysis, the whole raw data were normalized using the quantile normalization for the same overall distribution of the samples and the logarithm of normalized data to base 2 were calculated. Then, the distribution of intensity was checked by the histograms (Figure 2.3) and the distribution between the duplicated data at the same time point was tested by the scatter plots (Figure 2.4). As the distribution between duplicated data is similar, the slop of scatter plot was close to 1. By these histograms and scatter plots, three array data which were wild type at 36 h and BG11 at 36 h and 72 h were discarded because those data showed bad distributions and background noises were also removed from all data sets. Then, raw data were performed the quantile normalization again and the fold changes of mRNA expressions in BG11 compared with wild type in $\log_2$ scale were calculated. By NMF analysis, we obtained 10 major expression patterned clusters of mRNA and gene ontology data analysis was also performed using the 10 major clusters.

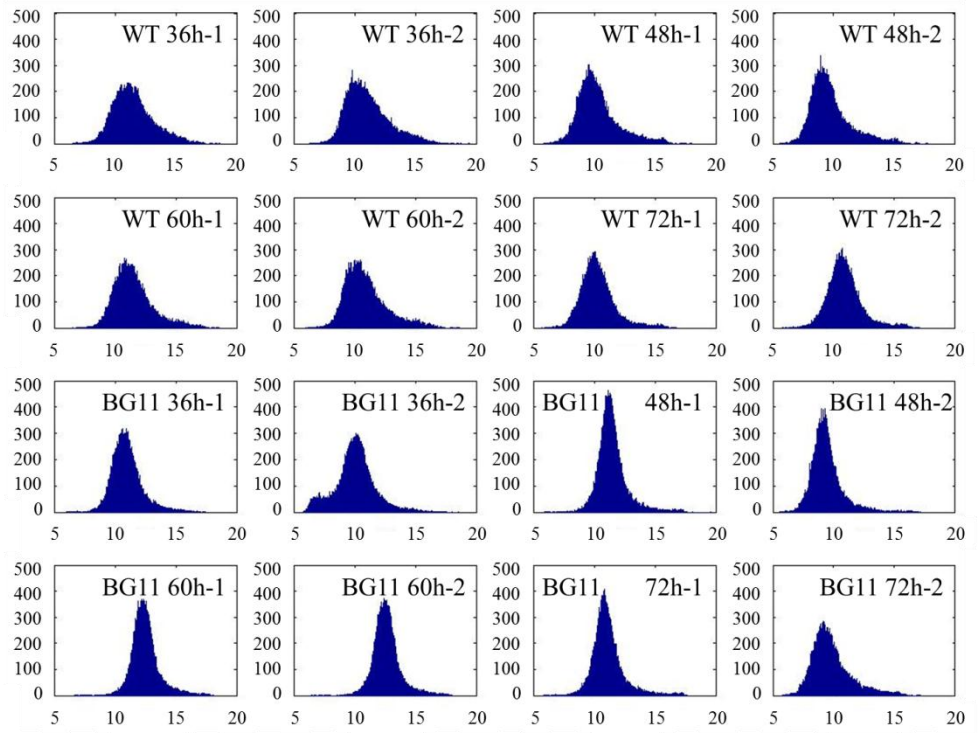


Figure 2.3 Histograms of raw data removed the background noises. Samples were duplicates of wild types (WT) and *ndgR* deletion mutants (BG11) at 36, 48, 60, and 72 h. X-axis: $\log_2$-fold-changes, Y-axis: number of genes.

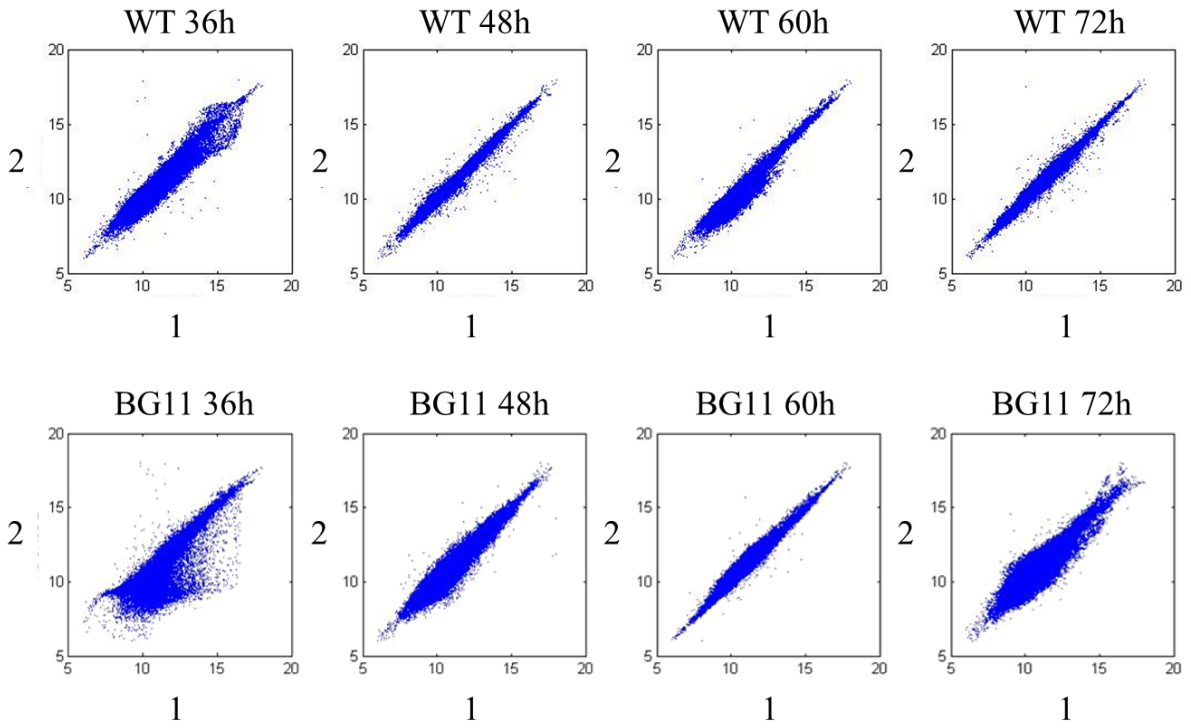


Figure 2.4 Scatter plots using the quantile normalized data which are removed background noises. X-axis and Y-axis were duplicated data at each time point.

2.8 Analysis of gene expression data of *absB* deletion mutants

We obtained gene expression datasets of *absB* deletion mutants in *Streptomyces coelicolor* (GSE4138) (Huang *et al.*, 2005) from the Gene Expression Omnibus (GEO) (Edgar *et al.*, 2002, Barrett *et al.*, 2007). In the absence (*absB*$^-$) and presence (*absB*$^+$) of *absB*, gene expression profiles were generated from the following four conditions in which *cdaR* in calcium dependent antibiotic (CDA) biosynthetic pathway, *actII-orf4* in actinorhodin (ACT) biosynthetic pathway, *redD* and *redZ* in undecylprodigiosin (RED) biosynthetic pathway were over-expressed, resulting in a total of eight datasets. The probe intensity data were $\log_2$-transformed and then normalized using the quantile normalization method (Bolstad *et al.*, 2003). Using the normalized data, for each probe, we calculated the $\log_2$-fold-changes (FCs) over time by subtracting the mean $\log_2$-intensity at the first time point (28 hr) from the median $\log_2$-intensities at the following time points were calculated in the *absB*$^-$ or *absB*$^+$ conditions. Based on the temporal FC profiles, we selected potential regulators controlled by AbsB as the regulators whose maximum and minimum FC values were below 1.5 in *absB*$^+$ conditions, but maximum FC values above 1.5 in *absB*$^-$ conditions. The potential regulators are listed in Table 3.1.

2.9 Analysis of gene expression data of *afsS* disruption mutants

Simplified scheme of data process and experimental procedure were described in Figure 4.1. The time-course microarray data were obtained in Gene Expression Omnibus (GEO) DataSets. The GEO accession numbers of wild type (M145) were GSE8084, GSE8086, GSE8107 and the GEO

accession numbers of the *afsS* disruption mutant were GSE8110, GSE8160 (Lian et al., 2008).

The raw intensities of each dataset were transformed to $\log_2$ scale and were normalized using quantile normalization method. When there were replicates in the same time point, median value of normalized intensities of replicates were selected. For each dataset, $\log_2$-fold-changes for each probe at each time point were computed by subtracting the intensity of first time point. The same process is done separately for all five datasets resulting in five fold-change matrix. Then, for each fold-change matrix, when there are multiple probes for a gene, the representative sum of fold-change across all time points for the gene was selected as that of the probe with the largest sum of $\log_2$-fold-change. In total, the five fold-change matrices were generated. Each of these five matrices was then transformed into a single vector. The five vectors for the five matrices were normalized using quantile normalization method to avoid a bias toward certain datasets with large fold changes. These vectors with normalized fold-changes were transformed back into the matrices. Finally, each of fold-change matrices were smoothed using moving average method (window size = 3).

To identify genes with differentially expressed pattern over the time-course in wild types, we applied the following statistical test to identify DEGs (differentially expressed genes), rather than performing multiple tests and then collectively considering the test results. The statistical method, a modified version of the time-course analysis method presented in Hwang et al. (Hwang *et al.*, 2005a, Hwang *et al.*, 2005b), includes a randomization-based null hypothesis distribution estimation for

adjusting multiple testing as follows: i) The forward and backward smoothed fold-change profile for each gene were numerically integrated over the time-course to obtain the area under the smoothed fold-change profile as a statistical measure; ii) To perform statistical hypothesis testing, a reference fold-change profiles was generated through the permutation based random sampling from the all five smoothed fold-change matrices; iii) A null hypothesis distribution was then empirically driven using trapezoidal method for the areas of reference fold-change profile; iv) P-values of area under forward and backward fold-change profiles for each gene were then calculated using null hypothesis distribution by one-tailed test of the corresponding area in step i) and minimum P-value was selected as significance of differential expression over time; v) Three P-values for each genes in wild types were combined using Stauffer's method. Finally, genes that show significantly altered expression pattern over the time-course in all three datasets of wild types identified with combined P-value < 0.01.

To find *afsS* disruption independent genes, the five smoothed fold-change matrices were combined by matching the gene IDs in the individual matrices, resulting in a combined fold-change matrix, and hierarchical clustering of fold-change profiles for DEGs in three datasets of wild type was performed. Pearson correlation and ward method were used as distance metric and linkage method for clustering, respectively.

2.10 Antibiotics measurement

Using a spectrophotometry, the amount of undecylprodigiosin (RED) and actinorhodin (ACT) was measured. Detail procedure followed the standard procedures. 1 ml of culture broth was sampled and 300 μL of culture

supernatants were mixed with 100 μL of 4N KOH for analyzing extracellular ACT and to analyse intracellular ACT, cell pellets were harvested from 400 μL culture broth by centrifugation at 13,000 rpm for 10 min, washed twice with distilled water and resuspended in 400 μL 1N KOH. Then the samples were centrifuged at 13,000 rpm for 5 min. Absorbance of resulted supernatant was measured at 640 nm (ε_{640}= 25320). Cell pellets from 0.4 mL culture broth were washed twice with distilled water and then RED was extracted with 1 mL of pH 2.0 methanol at 30 ℃ for 30 min. Cell pellets were removed after centrifugation at 13,000 rpm and the absorbance of resulted supernatant was measured at 530 nm for RED quantitation (ε_{530}= 100,500).

2.11 *In vitro* RNA cleavage assay

2.11.1 RNA transcripts and proteins preparation

Target genes were amplified by PCR using the primers containing the bacteriophage T7 promoter and genomic DNA of *Streptomyces coelicolor* A3(2) M145 as a template. Using the PCR products, RNA transcripts were transcribed in vitro at 37 ℃ for 6 hr in the buffer condition that was contained 40 mM Tris-HCl (pH 8.0), 8 mM $MgCl_2$, 2 mM spermidine, each 2.5 mM rNTP, 5 mM DTT, RNase inhibitor 20 Unit and T7 RNA polymerase 50 Unit. After that, transcripts were purified by urea PAGE and phenol/alcohol extraction and purification.

AbsB-His_6 was made by amplifying the coding region by PCR using genomic DNA of *Streptomyces coelicolor* A3(2) M145 as a template. AbsB-His_6 was cloned in the pET28a vector and expressed in *E. coli* BL21.

Over-expressed AbsB-His_6 was purified using Ni-NTA agarose beads and 20 mM imidazole buffer (50 mM phosphate buffer pH 8.0, 0.01% Tween 20, and 100 mM NaCl). Finally, purified AbsB-His_6 was eluted using 250 mM imidazole and calculated the concentration by Bradford assay.

2.11.2 *In vitro* RNA cleavage assay and image analysis

RNase cleavage assay was performed as the following condition (Amarasinghe *et al.*, 2001, Xu et al., 2008). Transcripts were regenerated by heating at 95 ℃ for 2 min and cooling on ice for 10 min. 1 μl transcripts, 1 μl AbsB-His_6, 9.8 μl reaction buffer (30 mM Tris-HCl (pH 8.0), 160 mM NaCl, 0.1 mM EDTA and 0.1 mM DTT) and 6 μl distilled H_2O were mixed and pre-incubated at 37 ℃ for 5 min. And 2 μl $MgCl_2$ (10 mM) which was pre-warmed at 37 ℃ were added to the reaction solution for starting the reaction. After incubation at 37 ℃ for 20 min, reaction stopped by adding EDTA-loading dye mixture. Then, whole transcripts were purified and concentrated by alcohol precipitation and analyzed by the BioRad Experion automated electrophoresis system.

2.12 Scanning Electron Microscopy (SEM)

The spores and hyphae of *Streptomyces* strains cultured on the solid media were observed by scanning electron microscopy (SEM) (JEOL JSM 5410LV, JAPAN). The razor-cut agar blocks were pre-fixed by 2% paraformaldehyde and 2% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.2) and post-fixed by 1% osmium tetroxide in 0.05 M sodium cacodylate buffer (pH 7.2) for 2 h, and then dehydrated and dried. Each

sample was gold sputter-coated and examined by SEM.

2.13 Transmission Electron Microscopy (TEM)

Cells grown in liquid minimal media were harvested by centrifugation at 13,000 rpm and observed by transmission electron microscopy (TEM) (JEM-1010, JEOL, Japan). Harvested cells were pre-fixed in 2% paraformaldehyde and 2% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.2) for 2 h, and then post-fixed with 1% osmium tetroxide in sodium cacodylate buffer (pH 7.2) for 2 h. The samples were then stained in 0.5% uranyl acetate for overnight at 4 ◦C and dehydrated through an alcohol series (30%, 50%, 70%, 90% and 100% twice each for 30 min, respectively). The samples were treated in 100% propylene oxide twice and embedded in an Spurr's resin and polymerized at 70 ◦C for 24 h. The embedded blocks were sectioned using an ultramicrotome (MTX, RMC, USA) and the sections were mounted on copper grids. The structures of samples were examined using a TEM.

2.14 Cell viability test

To analyse viability, the ratio of live/dead cells was determined by staining nucleic acids with a combination of the green-fluorescent SYTO® 9 stain (480/500 nm) and the red-fluorescent PI (propidium iodide; 490/635 nm) (the excitation/emission maxima for these dyes). All dyes were obtained from Molecular Probes, Inc (Eugene). 200 μL of samples were mixed with 1 μL of 1:1 mixture of SYTO® 9 and PI and incubated for 15 min at dark place. The released fluorescence was measured using GeminiXPS

fluorimeter (MolecularDevices, Sunnyvale, Calif).

2.15 Fluorescence Microscopy

100 µl of cell suspension was incubated with 0.5 µL of 1:1 mixture of SYTO® 9 and PI and incubated for 15 min at dark place.and an aliquot of the cells were then visualized by confocal microscopy (LSM-710; Zeiss, Jena, Germany) using 40× (NA 1.3) Plan-Apochromate objectives and sequential acquisition of separate channels.

Chapter III

AbsB-dependent transcriptional regulators involved in antibiotic production

3.1 Identification of novel interaction between known global regulator and new regulator

Streptomyces, a Gram-positive soil bacterium, has complex life cycle and produces various secondary metabolites, such as industrial antibiotics and natural products used as medicine. In *Streptomyces*, complex biological networks have evolved to execute various cellular processes that affect the antibiotic production, such as cell differentiation and biosynthesis of the secondary metabolites. Thus, understanding of these networks delineating underlying mechanisms of antibiotic production is a long time-awaited issue in pharmaceutical industry. Antibiotic production involves multi-layered regulation, including transcription, translation, post-translation, nutrient and precursor supply and uptake, transporter regulation, and secretion. Among them, transcriptional regulation has been considered as the primary control of the expression of the gene clusters involved in antibiotic production (Gramajo et al., 1993, Perez-Llarena *et al.*, 1997).

Transcriptional regulatory networks (TRNs) are essential to understand the complex transcriptional regulation of the genes involved in antibiotic production. A number of transcriptional regulators, such as sigma factors (Potuckova *et al.*, 1995, Tan *et al.*, 1998, Paget *et al.*, 1998), quorum receptors (Joo *et al.*, 2007, Xu *et al.*, 2010b), sensor kinases and response regulators (Brian *et al.*, 1996), control hundreds of the genes, thereby affecting various cellular processes related to antibiotic production. ActII-ORF4 (Gramajo et al., 1993, Fernandez-Moreno *et al.*, 1991), RedD (Takano et al., 1992) and RedZ (White & Bibb, 1997) have been characterized as pathway-specific transcriptional regulators directly controlling expression of the gene clusters involved in actinorhodin (ACT)

and undecylprodigiosin (RED) biosynthesis. Several other transcriptional regulators control other cellular processes, such as DNA-binding (Hesketh et al., 2009), phosphorylation (McKenzie & Nodwell, 2007), and RNA cleavage (Xu et al., 2010c). Furthermore, known global regulatory systems, AfsR/S and PhoR/P, interact with each other via transcriptional regulation (Santos-Beneit et al., 2009, Santos-Beneit et al., 2011).

Several tools have been developed to identify transcriptional regulators and their targets. ChIP-on-chip identifies target genes that are regulated by a specific transcription regulator (den Hengst et al., 2010, Pullan *et al.*, 2011, Bibb *et al.*, 2012). On the other hand, DACA (DNA affinity capture assay) identifies novel transcriptional regulators that are bound to specific target DNAs (Park et al., 2009). Using the DACA, we previously identified NdgR as a nitrogen-source dependent growth regulator and further found that NdgR affects both antibiotic production and morphological changes (Yang et al., 2009). Recently, gene expression profiling of wild-types and mutants of a certain regulator has emerged as an alternative means for a global search of its targets. Comparative analysis of gene expression data from wild-types and mutants can provide a panel of potential regulators and their targets, which improves understanding of the TRNs for the antibiotic production.

Here, we analyzed time-course gene expression profiles obtained from both wild-type and AbsB deleted conditions (Huang et al., 2005). *absB* encodes an RNase III homolog that acts as a global regulator and affects antibiotic production and morphological differentiation (Adamidis & Champness, 1992, Aceti & Champness, 1998, Price et al., 1999). To understand the TRNs for antibiotic production, it is essential to identify

downstream regulators of AbsB that control diverse cellular processes related to antibiotic production. Using the time-course gene expression datasets, we identified a list of potential regulators controlled by *absB* under *cdaR*, *actII-orf4*, *redD*, or *redZ* over-expressed conditions. In the absence of *absB*, these regulators showed the changes in their expression levels at least in one of the four over-expressed conditions. The transcripts of these regulators could be directly cleaved by AbsB, or they could be regulated indirectly via other intermediate regulators controlled by AbsB, such as AdpA (Xu et al., 2010c). Among the potential regulators, we experimentally confirmed that two example regulators are directly and indirectly by AbsB, respectively, and then showed that they are involved in antibiotic production. Therefore, our approach provides a comprehensive list of novel regulators controlled by *absB* for understanding multi-layered regulatory networks delineating antibiotic production.

3.1.1 Identification of AbsB-targeted regulators

AbsB encoding an RNase III homolog has a double-strand specific ribonucleolytic cleavage activity. AbsB protein regulates its own gene expression, as well as expression of various other genes by the cleavage of RNA transcripts. The targets of AbsB show lower mRNA expression levels under the *absB*$^{+}$ condition, due to cleavages of their mRNAs by AbsB, than under the *absB*$^{-}$ condition. Based on this notion, we first obtained time-course gene expression profiles generated from *cdaR*, *actII-orf4*, *redD* and *redZ* over-expressed strains under both *absB*$^{+}$ and *absB*$^{-}$ conditions. We then identified potential regulators controlled by AbsB as the ones showing temporal changes in their mRNA expression levels at least in one of the

four over-expressed strains under *absB*$^{-}$ conditions, but no change under *absB*$^{+}$ conditions (see section 2.8 Analysis of gene expression data of *absB* deletion mutants in Chapter II). Using this method, we selected 150 regulators, among the 670 regulatory genes in the genome of *Streptomyces*. The 150 potential AbsB-targeted regulators (Table 3.1) include 29 regulatory genes in *cdaR* over-expressed condition, 79 regulatory genes in *actII-orf4* over-expressed condition, 49 regulatory genes in *redD* over-expressed condition, and 11 regulatory genes in *redZ* over-expressed condition. Most of these regulators and their functions have not been previously reported. Among them, only 17 regulators have been previously reported, including pleiotropic transcriptional regulators (AdpA (Higo *et al.*, 2012, Wolanski *et al.*, 2011) and GlnK (Waldvogel *et al.*, 2011)), a morphogenesis regulator (SsgR (Traag *et al.*, 2004)), a nitrogen metabolism related regulator (GlnR (Tiffert *et al.*, 2008)), and antibiotics production related regulators (SCO1712 (Lee *et al.*, 2010) and SCO6808 (Yang et al., 2008)).

3.1.2 AbsB-targeted regulators control transcription and RNA metabolic processes

To understand what cellular processes the Abs-targeted regulators are involved in, we performed functional enrichment analysis using DAVID software (Huang da *et al.*, 2009). The analysis showed that these regulators are mostly involved in transcription, RNA metabolic processes, signal transduction, carbohydrate and amino acid metabolism, and nitrogen compound biosynthetic process (Figure 3.1 and Figure 3.2). ACT and RED have been considered as the major antibiotics.

Table 3.1 List of the regulatory genes and their log_2-maximum-fold-changes (FCs) that are shown as different mRNA expression patterns according to the presence of AbsB. The FC values are log_2 scale. Asterisked genes were putative targets of AdpA.

***cdaR* over-expressed condition**

genes	function	Max log_2-FC in *absB*$^-$	Max log_2-FC in *absB*$^+$
sco0574	IclR-family regulatory protein	0.838365	0.214972
sco0582	transcriptional regulator	0.724376	0.11122
sco0724	transcriptional regulator	0.678882	0.190585
sco1119	AsnC-family transcriptional regulatory protein	0.647616	0.094634
sco1221	AsnC-family regulatory protein	1.01604	0.061557
sco1295*	AsnC-family transcriptional regulatory protein	0.644344	0.036713
sco1351	large transcriptional regulator	1.000915	0.076602
sco1410	GntR-family transcriptional regulator	0.662133	0.08707
sco2223	TetR-family transcriptional regulator	0.610212	0
sco2319	TetR-family regulatory protein	0.815044	0
sco2508	metal uptake regulation protein	0.888161	0
sco2792*	AraC-family transcriptional regulator	1.056878	0.1118
sco2811	regulator	1.085913	0.111499
sco3207	TetR-family transcriptional regulator (fragment)	0.738937	0.382649
sco3932	GntR-family transcriptional regulator	0.681101	0.160792
sco4159	transcriptional regulatory protein	0.816918	0.055592
sco4180	iron uptake regulatory protein	0.786702	0.300791
sco4190	GntR-family transcriptional regulator	0.731859	0.22058

sco5100	GntR-family regulatory protein	1.018994	0.229832
sco5170	TetR-family transcriptional regulator	0.838463	0.229183
sco5513	acetolactate synthase 3 regulatory subunit	1.464156	0.006865
sco5552[*]	regulator	0.829544	0.080556
sco5584	nitrogen regulatory protein P-II	0.80362	0
sco5968	*bldA*-regulated nucleotide binding protein	0.823321	0.173604
sco6823[*]	ArsR-family transcriptional regulator	0.632899	0.056869
sco7498	transcriptional regulator	0.789948	0.229559
sco7512	AraC-family transcriptional regulator	0.58695	0
sco1488	pyrimidine regulatory protein PyrR	4.434417	0.30226
sco6312	transcriptional regulator	0.989081	0

***actII-orf4* over-expressed condition**

genes	**function**	**Max log_2-FC in *absB*$^-$**	**Max log_2-FC in *absB*$^+$**
sco0282	DeoR family transcriptional regulator	1.042797	0.018409
sco0289	probable transcriptional regulator	1.179358	0
sco0296	transcriptional regulator	0.984928	0.00162
sco0302	TetR-family transcriptional regulator	0.969798	0.188109
sco0407	transcriptional regulator	0.767914	0.074787
sco0421	two-component response regulator	0.718935	0
sco0430	TetR family transcriptional regulator	1.061182	0.073376
sco0456	LacI family transcriptional regulator	0.972161	0
sco0485	TetR-family transcriptional regulator	0.794664	0
sco0622	TetR-family transcriptional regulator	0.678665	0
sco0925	LysR-family transcriptional regulator	0.930447	0.247433
sco0940	MarR-family regulatory protein	1.066317	0.068574
sco1039	ROK family regulatory protein	0.871409	0
sco1094	TetR-family regulatory protein	0.841751	0.015972

sco1186	LacI-family transcriptional regulator	0.946876	0
sco1200	regulatory protein	0.958715	0
sco1351	large transcriptional regulator	1.216325	0.111082
sco1447	ROK-family transcriptional regulatory protein	0.671097	0.01256
*sco1463**	transcriptional regulator	0.994978	0
sco1678	transcriptional regulator	1.163872	0.248741
sco1736	MarR-family transcriptional regulator	0.90389	0.066074
sco1745	two-component system response regulator	0.770749	0
sco1897	transcriptional regulator	0.725943	0.005194
sco2013	two-component system response regulator	1.358023	0
sco2094	regulatory protein	1.128034	0
sco2105	transcriptional regulatory protein	1.249151	0
sco2319	TetR-family regulatory protein	0.923908	0
sco2331	MarR-family transcriptional regulator	1.304632	0.063117
sco2442	GntR-family transcriptional regulator	1.061134	0
sco2450	serine/threonine protein kinase (regulator)	0.609578	0.018367
sco2475	LysR-family transcriptional regulator	0.828741	0
sco2657	ROK-family transcriptional regulatory protein	1.151494	0.14249
sco2686	LuxR-family transcriptional regulator	0.710157	0.001183
sco2794	LacI-family transcriptional regulatory protein	0.654567	0.066262
sco2811	regulator	1.392869	0
sco2840	LysR-family transcriptional regulatory protein	0.892427	0.018888
*sco2845**	GntR-family transcriptional regulatory protein	1.240379	0.018089
sco2875	MerR-family transcriptional regulator	0.710051	0
sco3424	regulator	1.218599	0.005327
sco3447	transcriptional regulatory protein	1.219888	0
*sco3522**	transcriptional regulator	0.981081	0.281375
sco3691	regulatory protein	0.635341	0.035426
sco3696	transcriptional regulator	1.116768	0
sco3741	response regulator	0.842208	0
sco3914	transcriptional regulator	0.79728	0

sco3925	transcriptional regulator	1.03477	0
sco3932	GntR-family transcriptional regulator	0.956605	0
sco4167	TetR-family transcriptional regulator	0.876629	0.03865
sco4263	transcriptional regulatory protein	0.916952	0
sco4668	two-component system response regulator	0.635261	0
sco5132	two-component system response regulator	0.880373	0
sco5455	two-component system response regulator	1.031576	0
sco5463	MerR-family transcriptional regulator	0.992269	0
sco5528	transcriptional regulator	0.717917	0
sco5656	transcriptional regulatory protein	0.842433	0
sco5825	two-component response regulator	0.805769	0
sco5847	transcriptional regulator	0.888896	0.012372
sco5872	turgor pressure regulator	0.670579	0
sco5881	response regulator	0.76089	0.083795
sco5906	probable transcriptional regulator	1.163115	0
sco5956	regulatory protein	0.651341	0
sco6118	AraC family transcriptional regulator	0.61605	0.038219
sco6140	two-component system response regulator	0.783744	0.180217
sco6233	transcriptional regulator	0.593139	0.000274
sco6294	GntR-family regulatory protein	1.013924	0.185697
sco6599	transcriptional regulator	0.751695	0
sco6600	transcriptional regulator	1.225268	0.027228
sco6667	two-component regulator	0.796798	0.147709
sco6669	transcriptional regulator	1.029412	0
sco6792	TetR-family transcriptional regulator	1.193912	0
sco6801	LysR-family transcriptional regulator	0.892561	0
sco6823[*]	ArsR-family transcriptional regulator	1.028587	0
sco7498	transcriptional regulator	0.8709	0.100542
sco7552	TetR-family transcriptional regulator	0.700316	0
sco7809	TetR-family transcriptional regulator	0.608112	0
sco0344	GntR family DNA-binding regulator	0.863477	0.022225

sco0840	MarR-family transcriptional regulator	0.957221	0
sco1070	two component system response regulator	0.814642	0.050627
sco7014	probable LacI-family transcriptional regulatory protein	1.066922	0

redD over-expressed condition

genes	function	Max log_2-FC in ***absB***$^-$	Max log_2-FC in ***absB***$^+$
sco0298	LysR-family transcriptional regulator	0.606825	0
sco0310	TetR-family transcriptional regulator	0.584432	0
sco0428	TetR family transcriptional regulator	0.717595	0
sco0574	IclR-family regulatory protein	1.226381	0.224834
sco0728	regulatory protein	1.172415	0.291201
sco0806	LacI-family transcriptional regulatory protein	0.699281	0.311661
sco1043	transcriptional regulatory protein	0.85284	0.236132
sco1220	regulatory protein	1.038348	0.178445
sco1309	regulatory protein	0.677277	0.206399
sco1642	LacI family regulator	0.626507	0.250422
sco1712	TetR-family transcriptional regulator	0.677889	0
sco1718	regulator	0.582224	0.034336
sco1839*	transcriptional regulator	0.635941	0.045684
sco1872	IclR-family transcriptional regulator	0.973885	0.243827
sco2358	two-component system response regulator	1.003739	0
sco2442	GntR-family transcriptional regulator	0.746923	0.175556
sco2869*	regulatory protein	0.651106	0.031215
sco3018	regulatory protein	0.915516	0.038032
sco3066	regulator of Sig15	1.132027	0.072497
sco3259	KorSA, regulatory protein	0.903098	0
sco3447	transcriptional regulatory protein	1.066975	0

sco3571	transcriptional regulator	0.664429	0
sco4116	AfsR-like regulatory protein	0.786408	0.238499
sco4313	regulatory protein	0.779636	0.075638
sco4358	TetR family transcriptional regulatory protein	0.812107	0.081191
sco4480	TetR-family transcriptional regulatory protein	1.076827	0
sco4499	TetR-family regulatory protein	0.890319	0
sco5103	regulatory protein	0.689939	0.259283
sco5170	TetR-family transcriptional regulator	0.760427	0.170949
sco5513	acetolactate synthase 3 regulatory subunit	1.643443	0.214267
sco5584	nitrogen regulatory protein P-II	0.921315	0.184113
sco5611	transcriptional regulator	0.698467	0.145118
sco5778	two-component system regulator	0.729192	0
sco5956	regulatory protein	0.702327	0.10171
sco6144	transcriptional regulator	0.819269	0.041123
sco6299	TetR-family transcriptional regulator	1.080585	0.529952
sco6808	ArsR-family transcriptional regulator	1.790511	0.466857
sco6823[*]	ArsR-family transcriptional regulator	1.087058	0.218131
sco7027	probable LacI-family transcriptional regulator	1.097458	0.042019
sco7075	two component response regulator protein	1.217565	0.086468
sco7097	MarR-family regulatory protein	0.893583	0
sco7137	regulatory protein	0.62174	0.171435
sco7222	TetR-family transcriptional regulator	1.15518	0.390734
sco7351	AraC-family transcriptional regulator	0.863691	0
sco7424	MarR-family transcriptional regulator	1.084452	0.08336
sco7712	two component system response regulator	0.607564	0.326216
sco7780	transcriptional regulator	0.877609	0
sco6121	transcriptional regulator	0.637816	0.245777
sco7543	Crp-family transcriptional regulator	1.058461	0.104461

***redZ* over-expressed condition**

genes	function	Max log_2-FC in *absB*$^-$	Max log_2-FC in *absB*$^+$
sco0574	IclR-family regulatory protein	0.824938	0
sco1897	transcriptional regulator	0.651208	0
sco1956	LacI-family transcriptional regulator	0.600506	0
sco2745	probable LacI-family transcriptional regulator	0.661369	0.335559
sco2775	TetR-family regulatory protein	0.612057	0.321213
sco2845	GntR-family transcriptional regulatory protein	0.586526	0.244959
sco3269	GntR-family regulator	0.956509	0
sco3932	GntR-family transcriptional regulator	0.631907	0.172253
sco4194	TetR-family transcriptional regulator	0.617811	0
sco5678	regulatory protein	0.735924	0.220819
sco5758	transcriptional regulator	0.609411	0.007051

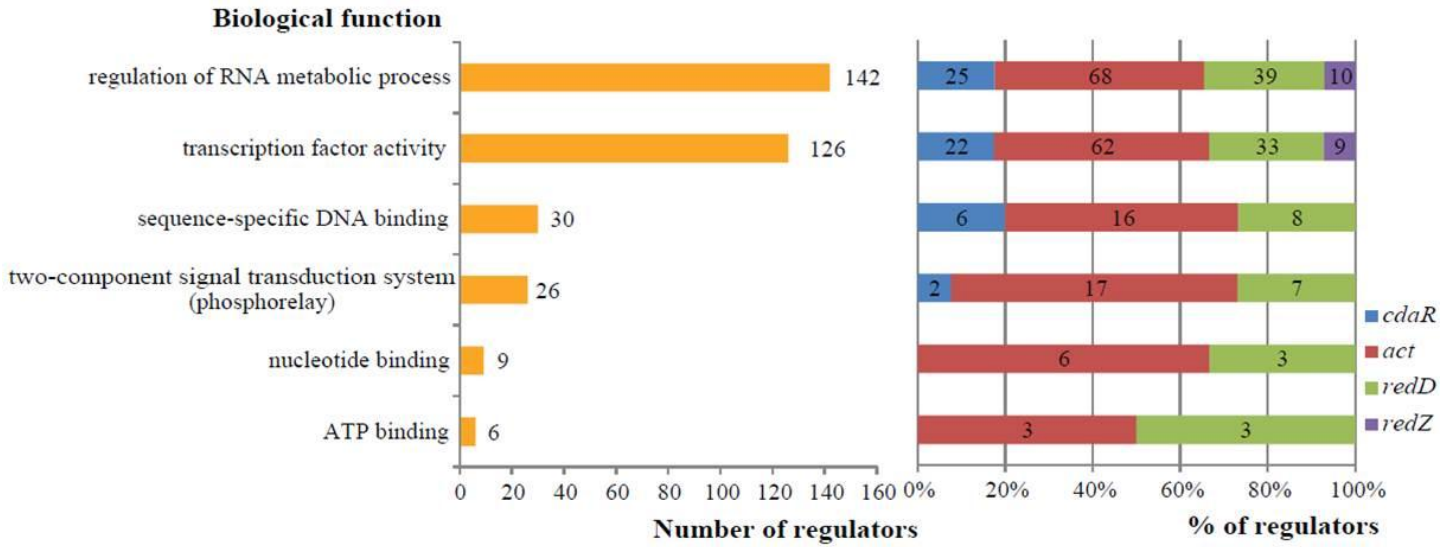


Figure 3.1 Cellular processes represented by the 150 potential regulators. Left panel, the total count of regulators involved in six GO biological processes (GOBPs); and Right panel, the percent of the regulators involved in the six GOBPs in *cdaR* (blue), *actII-orf4* (red), *redD* (green), and *redZ* (purple) over-expressed strains. Labels on the stacked bars in the right panel denote the counts of the regulators identified from the individual strains.

For the 79 regulators identified under *actII-orf4* over-expressed condition, their functions are mostly unknown and thus their association with ACT production is not clear. In contrast, the 49 regulators identified under *redD* over-expressed condition include several known regulators related to RED production. Thus, we focused on the 49 regulators that are likely to be associated with RED production (Table 3.1). To experimentally test the validity of the 49 regulators as AbsB targets, we chose two example regulators, *sco6808* most up-expressed regulatory genes in the *absB*$^-$ condition and *sco1839*, one of the lowest expressed regulatory genes. Their contributions to ACT and RED production have been previously shown (Yang et al., 2008). However, it has not been reported whether they are direct targets of AbsB. We first examined whether these two regulators regulate ACT and RED production. We previously showed that antibiotic production was accelerated by deletion of *sco6808* and decreased remarkably by over-expression of *sco6808* in R5$^-$ complex media. We newly measured RED and ACT production in the absence of *sco1839*. The deletion of *sco1839* decreased both of ACT and RED production in R5$^-$ complex media (Figure 3.3). These data indicated that they control the antibiotic production as AbsB downstream regulators.

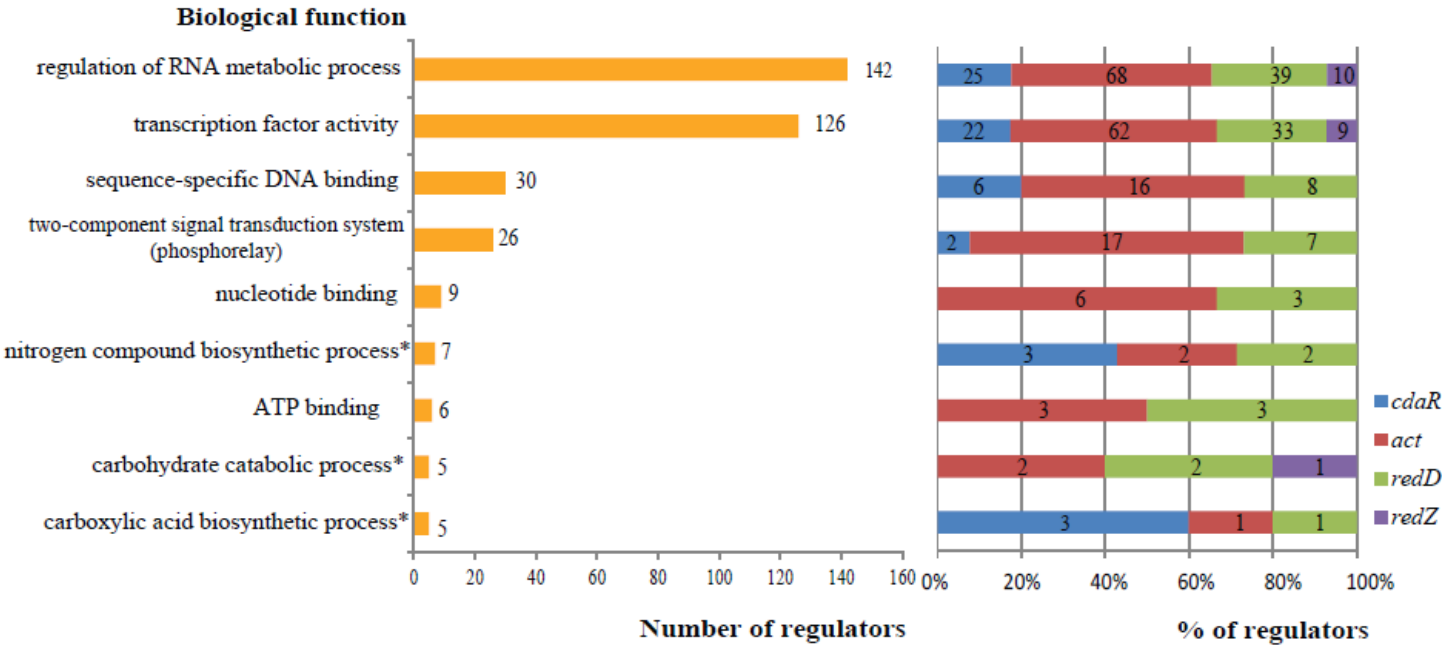

Biological function
regulation of RNA metabolic process
transcription factor activity
sequence-specific DNA binding
two-component signal transduction system (phosphorelay)
nucleotide binding
nitrogen compound biosynthetic process*
ATP binding
carbohydrate catabolic process*
carboxylic acid biosynthetic process*
142
126
30
26
9
7
6
5
5
0
20
40
60
80
100
120
140
160
Number of regulators
25 68 39 10
22 62 33 9
6 16 8
2 17 7
6 3
3 2 2
3 3
2 2 1
3 1 1
0%
20%
40%
60%
80%
100%
% of regulators
cdaR
act
redD
redZ

Figure 3.2 GOBP analysis of the 150 potential regulators and their orthologs in *E. coli* K12. In this analysis, we identified the orthologs of the 150 regulators in *E. coli* and added the GOBPs of the orthologs to identify the GOBPs represented by the 150 regulators. Since GOBP information in *E. coli* is more extensive than that in *Streptomyces*, this integrative analysis can reveal further accurate GOBPs represented by the 150 regulators. Among the 150 regulators, 48 were mapped to orthologous genes in *E. coli* (6, 25, 13, 4 genes in *cdaR*, *actII-orf4*, *redD*, and *redZ* over-expressed strains, respectively) using BLAST search. With the integration of the GOBPs of the orthologs in *E. coli*, we could identify three additional GOBPs, denoted by asterisks. Left panel, the total count of the regulators involved in the nine GOBPs; and Right panel, the percentage of the regulators involved in the six GOBPs in *cdaR* (blue), *actII-orf4* (red), *redD* (green), and *redZ* (purple) over-expressed strains. Labels on the stacked bars in the right panel denote the counts of the regulators identified from the individual strains.

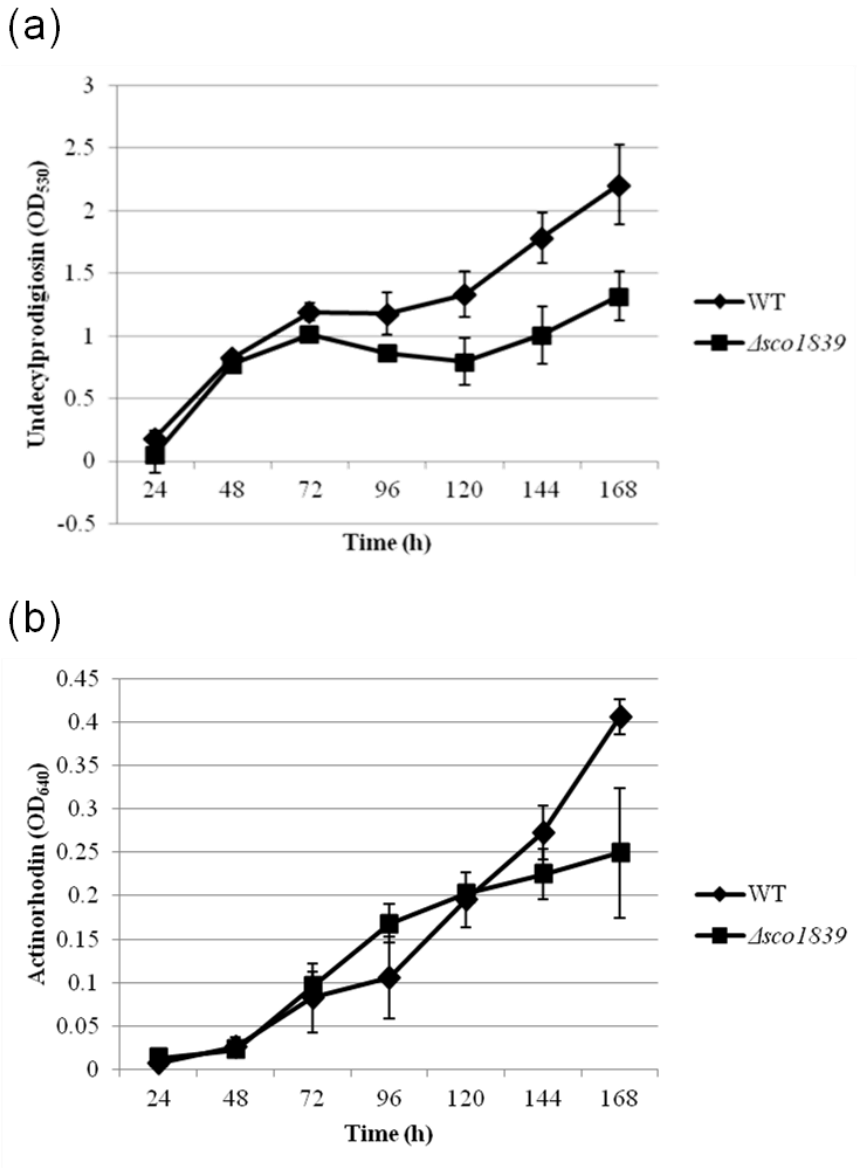


Figure 3.3 Antibiotics production when *sco1839* was deleted. RED production (a) and ACT production (b) are decreased in *sco1839* deletion mutant in $R5^-$ complex media.

3.1.3 SCO6808 controls antibiotic production as a direct downstream target of AbsB

Sco6808 encodes a putative ArsR-family transcriptional regulator. This gene has 139 bp of 5' UTR of *sco6808* between *sco6808* and *sco6809*. This 5' UTR may contain regulatory elements controlling expression of *sco6808*. Thus, there could be other regulators that recognize these elements and control expression of *sco6808*. According to the gene expression data, the genes located around *sco6808* showed no significant changes in their mRNA expression levels between the *absB*$^{-}$ and *absB*$^{+}$ conditions. In *redD* over-expressed condition (Figure 3.4), *sco6808* is up-regulated in the *absB*$^{-}$ condition, but the other genes showed no differential expression depending on the presence of *absB*, indicating the possibility that *sco6808* is directly regulated by AbsB. To confirm this possibility, we performed *in vitro* RNA cleavage assay using purified AbsB-His_6. The activity of AbsB-His_6 was checked by the cleavage of the *absB* transcripts, which were previously shown to be the direct target of AbsB (Figure 3.5). 553-nucleotide (nt) *sco6808* including 5' UTR was transcribed *in vitro* (Figure 3.6 (a)). When the RNA transcripts were reacted with AbsB-His_6, 533-nt transcripts were cleaved into two transcripts near 200-nt and 300-nt (Figure 3.6 (b)), showing that *sco6808* is a new downstream regulator of AbsB. We previously showed that SCO6808 is a repressor of antibiotic production (Yang et al., 2008). Thus, the data collectively showed that AbsB, as a higher level regulator, negatively regulates *sco6808*, a negative regulator of antibiotic production through the ribonucleolytic cleavage, leading to positive regulation of antibiotic production.

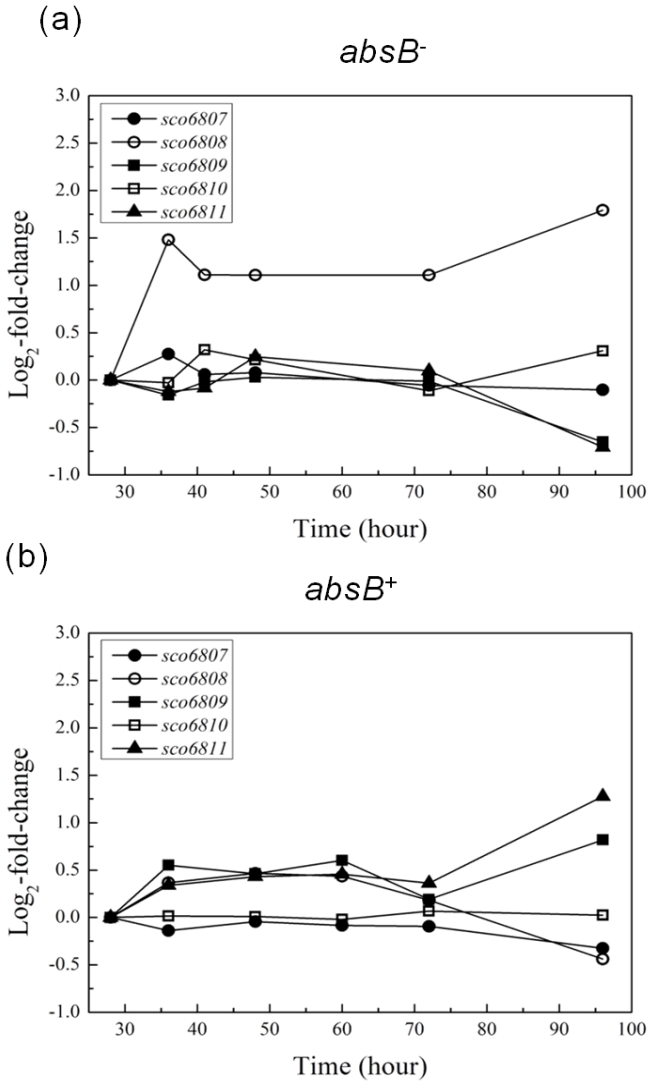


Figure 3.4 Fold change patterns of genes from *sco6807* to *sco6811* in the condition of *absB*$^{-}$ (a) and the condition of *absB*$^{+}$ (b). *Sco6808* shows dramatic increase in mRNA expression in *absB*$^{-}$, but other genes do not show big differences. And in *absB*$^{+}$ condition, genes change their expression pattern by the regulation of AbsB.

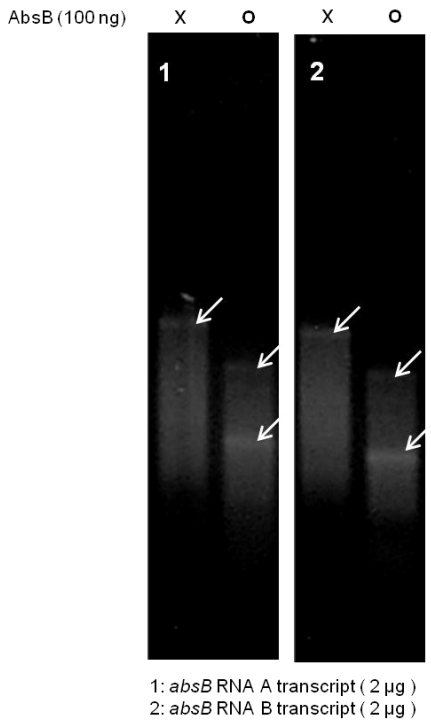


Figure 3.5 The activity of AbsB-His_6 was checked by the cleavage of *absB* own RNA transcripts, which were known to the target of AbsB. AbsB-His_6 was performed properly that known targets were cleaved by AbsB-His_6 as Xu et al. previously reported (Xu et al., 2008). *AbsB* RNA transcripts were obtained by *in vitro* transcription using genomic DNA of *S. coelicolor* and primers contained the bacteriophage T7 promoter. The primer sequences are RNA A-F (5'-TAA TAC GAC TCA CTA TAG GGC CGG ATT GGG CCG AGA GCG AAT GG-3'), RNA A-R (5'-GCG GTC TGG GCC GGT GGA TGA GCG-3'), RNA B-F (5'-TAA TAC GAC TCA CTA TAG GGG GCT CTT CGG TCG CGT GTA TGC C-3') and RNA B-R (same as RNA A-R). *In vitro* RNA cleavage assay was performed as mentioned in Materials and Methods.

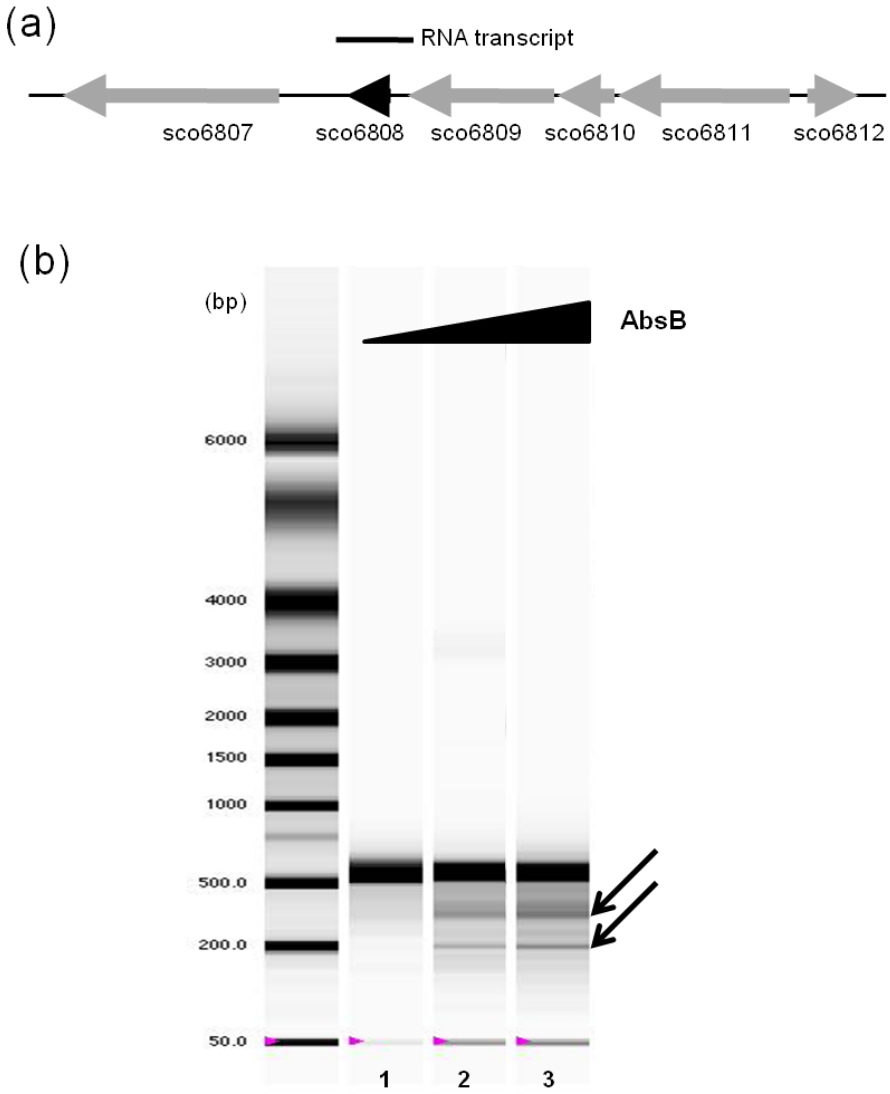


Figure 3.6 Cleavage of *sco6808* transcripts by AbsB-His_6. (a) The location of genes and transcripts. (b) *In vitro* ribonucleolytic cleavage assay of *sco6808*. Two transcripts resulted from the cleavage are indicated by the arrows. Lane 1, transcripts without AbsB; Lane 2, transcripts with 95 μg of AbsB-His_6; Lane 3, transcripts with 379 μg of AbsB-His_6.

3.1.4 SCO1839 controls antibiotic production as an indirect downstream target of AbsB

Sco1839 is a putative transcriptional regulator containing a helix-turn-helix motif. We tested whether it was a direct target of AbsB. The 632-nt *sco1839* were first transcribed *in vitro* including 337-nt 5' UTR, and the transcripts were reacted with AbsB-His_6 (Figure 3.7 (a)). Unlike the case of *sco6808*, the cleavages of the transcripts were not observed (Figure 3.7 (b)), indicating that *sco1839* was not a direct target of AbsB, but is indirectly regulated by AbsB via an intermediate regulator. AbsB positively regulates the antibiotic production. Thus, the downstream repressors of AbsB, such as *sco6808*, which negatively regulate the target genes, should be negatively regulated by AbsB. In contrast, the positive regulator of antibiotic production, such as *sco1839*, should not be negatively regulated by AbsB as being a direct target of AbsB. A possible link between AbsB and *sco1839* is through the relationships between *sco1839* and AdpA, a pleiotropic transcriptional regulator. Recently, Xu el al. reported that *adpA* is directly regulated by AbsB and AdpA also control AbsB by the activation of intracellular proteases, thereby forming a feedback regulatory loop (Xu et al., 2010c). They reported a list of the genes which had the putative AdpA binding motifs, including *sco1839*, indicating that *sco1839* is indirectly regulated by AbsB through AdpA. Thus, the data collectively showed that AbsB, as a higher level regulator, negatively regulates AdpA by its ribonucleolytic cleavage activity, leading to positive regulation of *sco1839* and antibiotic production.

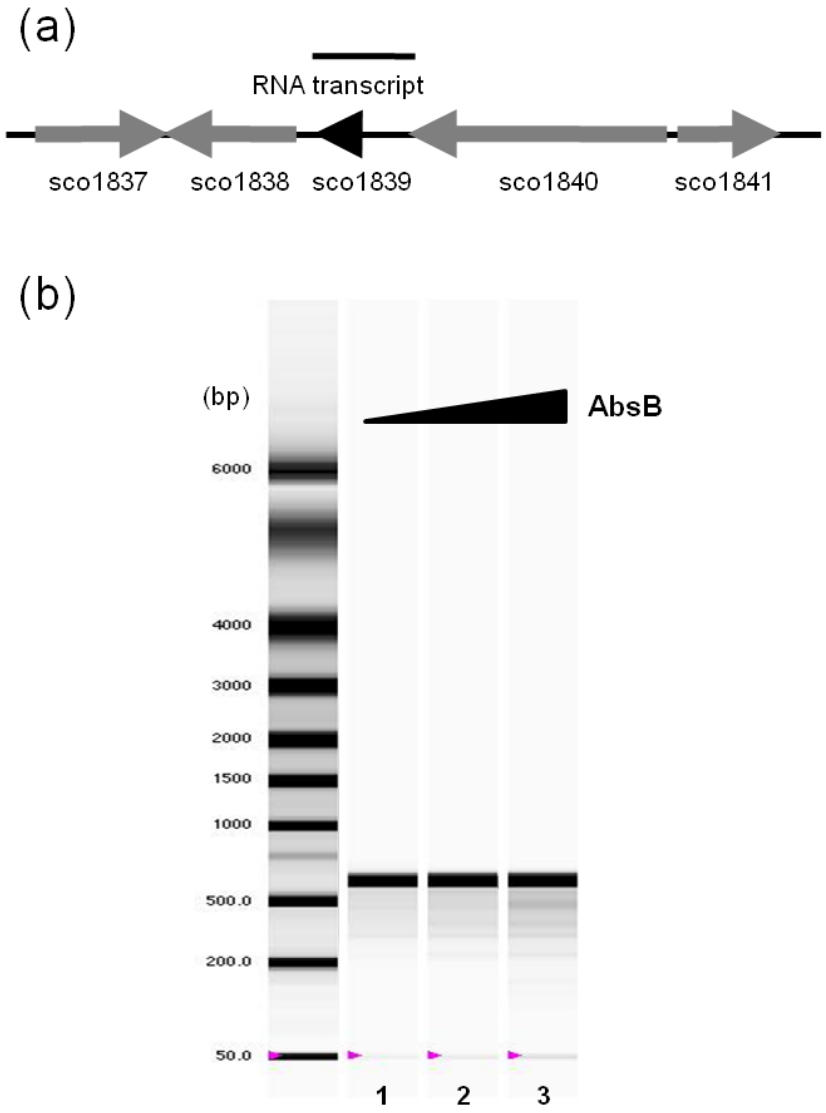


Figure 3.7 Cleavages of *sco1839* transcripts by AbsB-His_6. (a) The location of genes and transcripts. (b) *In vitro* ribonucleolytic cleavage assay of *sco1839*. Lane 1, transcripts without AbsB; Lane 2, transcripts with 95 μg of AbsB-His_6; Lane 3, transcripts with 379 μg of AbsB-His_6.

3.2 Conclusion

Transcriptional regulatory networks (TRNs) delineating antibiotic production in *Streptomyces coelicolor* still remain elusive. Due to the lack of a tool to systematically investigate the regulators involved in biosynthesis of secondary metabolites, only a limited number of regulators has been characterized. In this study, we proposed a systematic approach that provides 150 potential regulators controlled by AbsB, which can control antibiotic production by analyzing gene expression profiles collected under *cdaR*, *actII-orf4*, *redD*, or *redZ* over-expressed conditions. Among the 150 AbsB-targeted regulators, we experimentally verified the roles of *sco6808* and *sco1839* in regulating antibiotic production, which extends current understanding of a TRN delineating RED production (Figure 3.8). Experimental tests of this comprehensive list of these potential regulators and further studies of the interrelationships among them would extend extensively the current TRN. As demonstrated, the proposed approach is highly effective to screen the downstream regulators of a high level regulator, compared to conventional genetic screenings. This approach can be applied to various problems where TRNs should be decoded to generate a comprehensive list of novel regulators, providing a basis for understanding multi-layered TRNs.

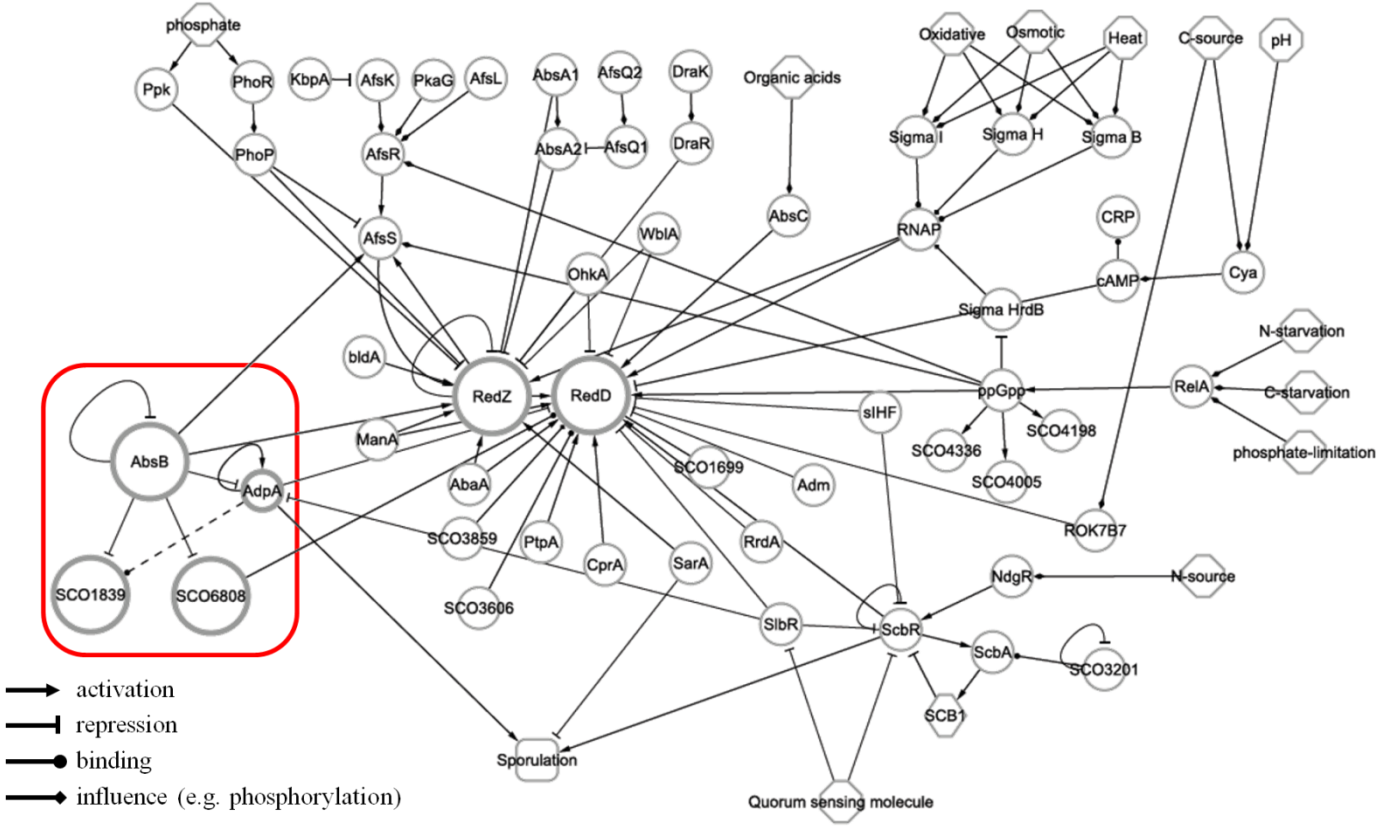


Figure 3.8 Extension of the current transcriptional regulatory network for RED production. Newly extended network is boxed by round rectangle.

Chapter IV

Antibiotic overproduction by the combination of transcriptionally independent regulators

4.1 Effective transcriptional control of antibitotics production

Streptomyces is well known genus of actinobacteria involving a complex secondary metabolism. Most pharmaceutically useful secondary metabolites such as antibiotics are produced by streptomycetes. In spite of the importance of secondary metabolites, it is still challenging to increase their production through chemical synthesis, due to complex structures of the secondary metabolites. Therefore, production of secondary metabolites using *Streptomyces* as host strains has been extensively endeavored. Various approaches for increasing production of secondary metabolites have been developed. First, a pool of cofactors or precursors in host cells one of them, such as CoA derivatives, the precursors of many antibiotics, has been increased, leading to increased antibiotics production (Guilfoile & Hutchinson, 1991, Murakami et al., 2011, Tahlan et al., 2007). Second, important genes or gene clusters related with the secondary metabolites biosynthesis have been introduced into host cells by genetic engineering. Recently, many antibiotics were produced by the heterologous expression (Park et al., 2011, Qiu et al., 2011) and by the insertion of the multi-copy antibiotics biosynthetic gene cluster (Murakami et al., 2011, Yanai et al., 2006). Other approaches involve the substitution of the specific promoter (Blount et al., 2012, Guo et al., 2012, Jensen & Hammer, 1998), controlling unwanted pathways (Kurumbang et al., 2010, Li & Townsend, 2006), to promote the resistance of cytotoxicity by over-expressing antibiotics resistant genes or efflux pumps (Dairi et al., 1995, Malla et al., 2010, Yanai et al., 2006), engineering the global or pathway-specific regulators (Adamidis & Champness, 1992, Bruheim et al., 2002, McKenzie & Nodwell, 2007, Takano et al., 1992, Uguru et al., 2005) and so on.

In *Streptomyces*, complicated transcriptional regulatory networks control the secondary metabolism (Martin & Liras, 2010). Transcriptional regulators control collectively the expression of several target genes related to antibiotics production. Therefore, engineering transcriptional regulators involving their over-expression or deletion is an effective approach to increase the production of secondary metabolites. Over the decades, many efforts have been taken to understand the transcriptional regulatory network underlying the secondary metabolism in *Streptomyces*. Using the conventional engineering of transcriptional regulators, several pathway-specific regulators controlling certain pathways related to the secondary metabolism, such as ActII-ORF4 in actinorhodin (ACT) biosynthetic gene cluster (Fernandez-Moreno et al., 1991), RedD in undecylprodigiosin (RED) gene cluster (Takano et al., 1992) and CdaR in calcium-dependent antibiotic (CDA) gene cluster (Ryding et al., 2002), have been identified. Moreover, a number of putative transcriptional regulators have been identified using microarrays (Pullan et al., 2011), 2-D electrophoresis (Wang *et al.*, 2010, Yang et al., 2009) and DNA affinity capture assay (DACA) (Park et al., 2009, Yang et al., 2009). These approaches revealed many transcriptional regulators related to antibiotics production, thus improving understanding of the complex transcriptional regulatory networks. However, it is still far from detailed understanding involving major regulators and their relationships to stratify an effective scheme to optimally increase antibiotics production. For instance, PhoP and AfsR related to phosphate control metabolism and secondary metabolism are possible to cross-regulate their lower level regulators. Pathway-specific regulators, such as ActII-OFR4 and RedD, also control other structural

genes and regulatory genes (Huang et al., 2005, Martin & Liras, 2010, Santos-Beneit *et al.*, 2012).

Many transcriptional regulators have been manipulated to increase antibiotics production. AfsS, a 63 amino-acid-protein, has been characterized as a global regulator activating antibiotics biosynthetic gene clusters (Lee et al., 2002, Vogtli et al., 1994). Genome-wide transcriptome analysis using microarrays revealed that AfsS modulates various cellular processes, including the nutrient starvation response and antibiotics biosynthesis gene clusters (Lian et al., 2008). Over-expression or deletion of multiple transcription regulators, rather than single regulators, which control non-redundant pathways related to antibiotics production, would effectively increase the antibiotics production. However, there has been no systematic approach to identify these transcriptional regulators. Herein, we identified the combination of two transcriptional regulators controlling the antibiotics related pathways independently of AfsS using gene expression profiles of *afsS* disruption mutants and then experimentally demonstrated that we dramatically increased antibiotics production in *Streptomyces coelicolor* by the combination of *sco4677* with *afsS* and *phoU* with *afsS*. Therefore, the proposed approach to identify and manipulate transcriptional regulators controlling independent pathways provides an effective strategy to increase antibiotics production, as well as a basis for enhancing understanding of a transcriptional network for the antibiotics production.

4.1.1 Identification of AfsS independent regulators

AfsS, a 63 amino-acid-protein, has been characterized as a global regulator

activating antibiotics biosynthetic gene clusters (Lee et al., 2002, Vogtli et al., 1994). Comparative analysis of gene expression profiles of *afsS* disruption mutants and wild types can reveal the genes affected by AfsS. We first obtained three time-course array datasets of wild types and two of *afsS* disruption mutants in *Streptomyces coelicolor* from the Gene Expression Omnibus (GEO) database (Barrett et al., 2007, Edgar et al., 2002). We then identified AfsS regulated genes as the genes whose expression levels were significantly ($P<0.01$) changed in the mutants, compared to wild types, using the previously reported method (see section 2.9 Analysis of gene expression data of *afsS* disruption mutants in Chater II).

Transcription regulators controlling antibiotics related pathways independent of AfsS can be used to further increase the antibiotics production together with AfsS. To identify these AfsS independent regulators, we searched for the regulators that show similar mRNA expression patterns between wild types and *afsS* disruption mutants using the scheme shown in Figure 4.1. To this end, in each dataset, we first computed the $\log_2$-fold-changes over time using the first time point as a reference and then identified the genes showing significant ($P<0.01$) differential expression patterns over time in wild types. Finally, we clustered gene expression patterns of these genes and identified AfsS independent genes as the genes in two clusters showing similar expression patterns between wild types and *afsS* disruption mutants (Figure 4.2). 109 genes were up-regulated in both wild types and *afsS* disruption mutants while 211 genes were down-regulated.

These AfsS independent genes included 11 up-regulated regulators

(i.e. 10 transcriptional regulators and a sigma factor) and two down-regulated transcriptional regulators. These regulators are listed in Table 4.1. These regulators can be upstream regulators of AfsS or downstream regulators belonging to the pathways that are not controlled by AfsS. Normally, mRNA expressions of transcriptional regulators which were especially related with secondary metabolism were dramatically changed during growth phage, and we supposed that is the reason why the pathway-specific regulators such as *cdaR* and *redD* showed in the candidate list since their expression patterns did not changed much although the expression levels were changed. Our goal was to increase the antibiotics production by manipulating some of these regulators. Thus, we focused on the 11 up-regulated AfsS independent regulators.

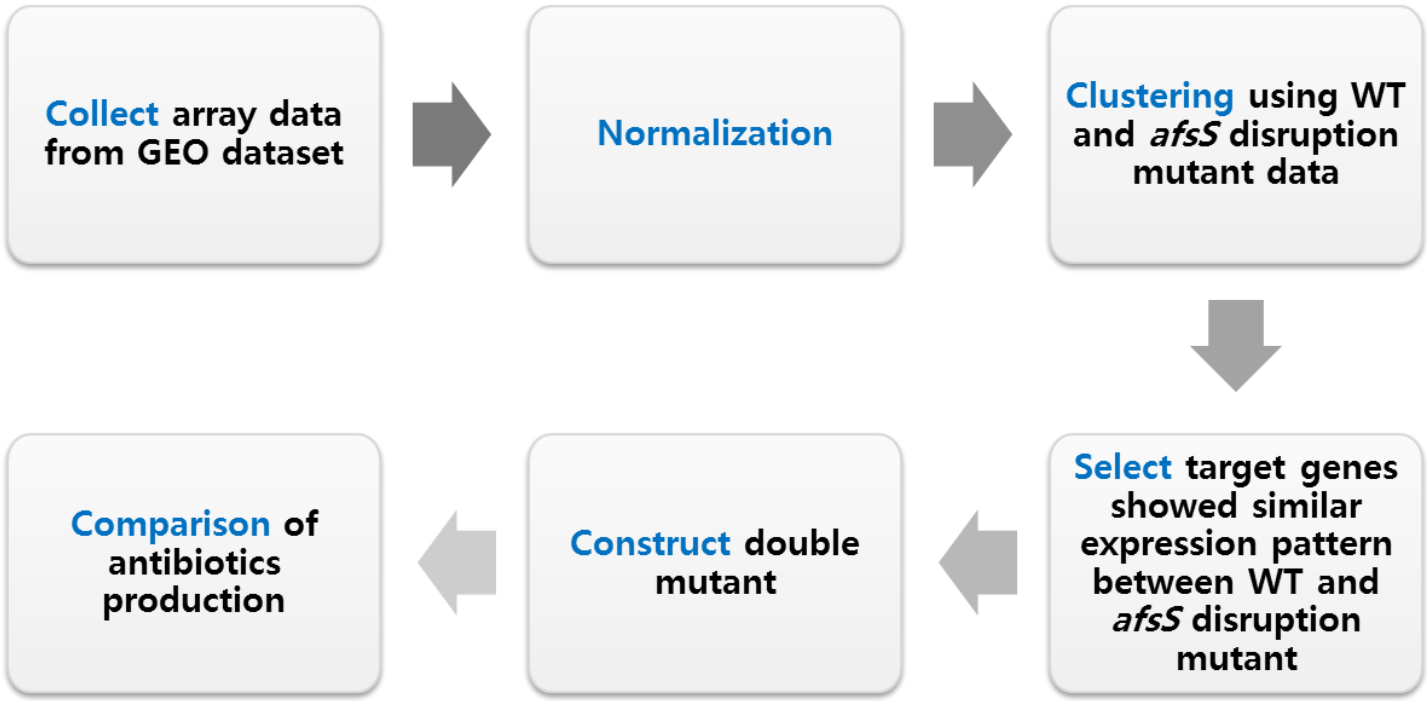


Figure 4.1 Scheme of data process and experimental procedure.

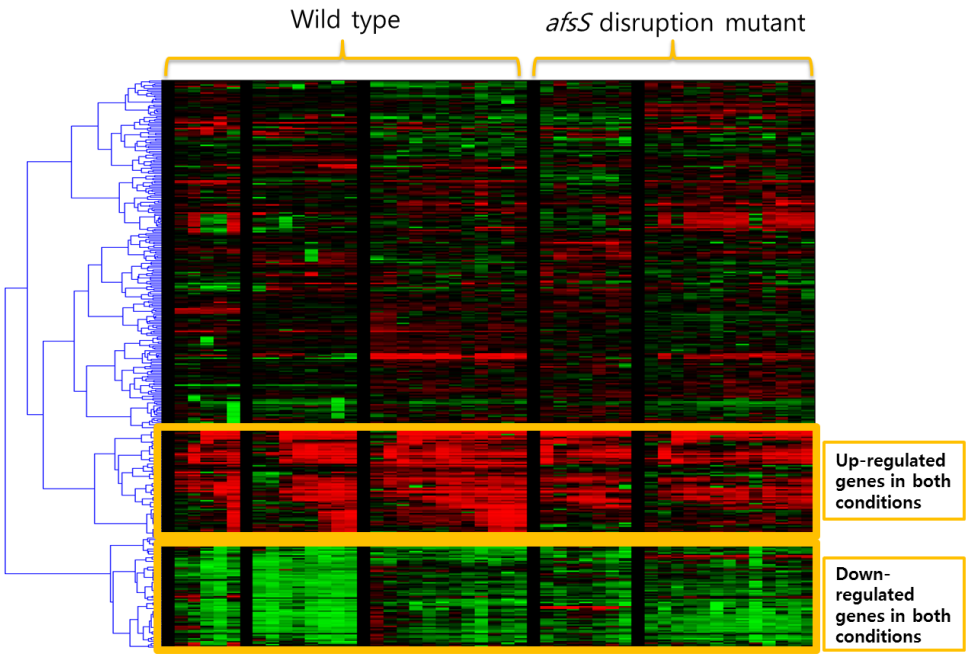


Figure 4.2 Clustering of mRNA expression in wild types and *afs*S disruption mutants. Differentially expressed genes identified in wild types were also found in the condition of *afs*S disruption mutants. Up- and down-regulated genes in both conditions were categorized by clustering.

Table 4.1 Regulators that were selected by clustering.

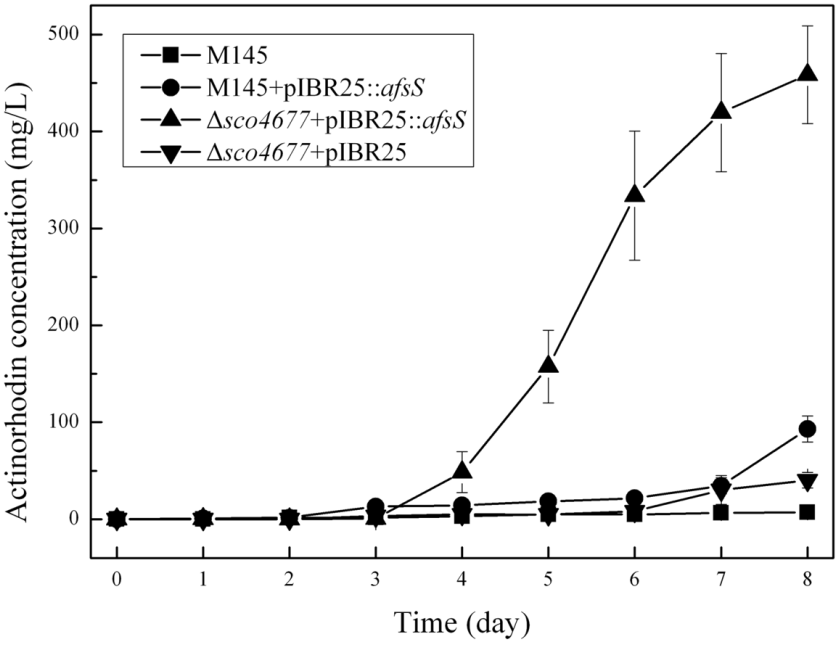
Gene		Function
Up-expressed patterned cluster		
sco0608		regulatory protein
sco1699		transcriptional regulator
sco1839		transcriptional regulator
sco3217	*cdaR*	transcriptional regulator
sco3323	*bldN*	RNA polymerase sigma factor
sco3579	*wblA*	regulatory protein
sco4228	*phoU*	phosphate transport system regulator
sco4677		antagonistic regulator of σ^F
sco4768	*bldM*	two-component regulator
sco5877	*redD*	transcriptional regulator
sco7252		regulatory protein
Down-expressed patterned cluster		
sco3068	*sigI*	RNA polymerase sigma factor
sco4180	*nur*	iron uptake regulatory protein

4.1.2 Double mutation of *sco4677* and *afsS* increased antibiotics production

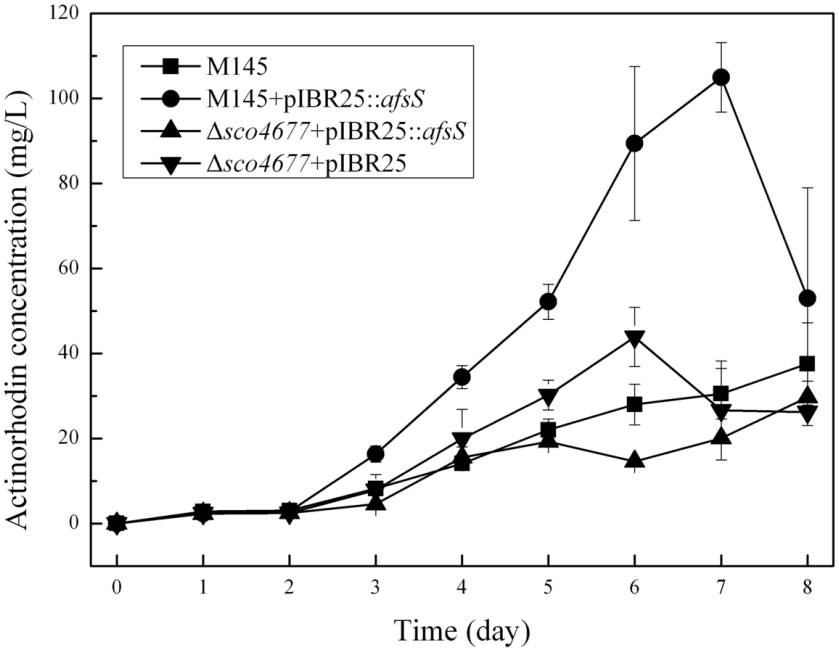
To demonstrate the validity of these AfsS independent regulators, we chose two regulators from the 11 up-regulated AfsS independent regulators and experimentally tested whether these regulators increase the production of antibiotics RED and ACT in the AfsS independent manner, leading to additional increases of RED and ACT production to that caused by AfsS. Among the 11 up-regulated AfsS independent regulators, we first selected *sco4677*, an antagonistic regulator of sigma factor F, which has a HATPase_c domain and regulates antibiotics production and morphological differentiation (Kim *et al.*, 2008). It was previously reported that antibiotics production were enhanced when the *sco4677* was deleted in R5$^-$ complex media. Therefore, we constructed the *afsS* over-expressed and *sco4677* deleted double mutants in *Streptomyces coelicolor*. Due to the independency between AfsS and SCO4677, they would not interfere with each other, leading to increasing the antibiotics production in a synergistic manner. Spores of M145, BGafsS (M145+pIBR25::*afsS*), BG4677 (Δ*sco4677*+pIBR25) and BG4677S (Δ*sco4677*+pIBR25::*afsS*) were germinated in R5$^-$ complex media, and the amounts of RED, intracellular ACT and extracellular ACT were measured over time (Figure 4.3). As expected, all the antibiotics productions were increased dramatically in double mutants: RED was increased by about 8 fold, and intracellular ACT was further increased by about 11 fold, compared with M145. BGafsS increased the antibiotics production, and ACT was fully exported from the inside of cells. Similar to BGafsS, M145 and BG4677 also exported the produced antibiotics, rather than accumulated them inside the cells. In

contrast, BG4677S did not secrete the produced antibiotics considerably. These data strongly indicate the synergistic effect of SCO4677 and AfsS, supporting that the two regulators independently act to increase the antibiotics production.

(a)


M145
M145+pIBR25::afsS
Δsco4677+pIBR25::afsS
Δsco4677+pIBR25
Actinorhodin concentration (mg/L)
Time (day)
0
100
200
300
400
500
0
1
2
3
4
5
6
7
8

(b)


M145
M145+pIBR25::afsS
Δsco4677+pIBR25::afsS
Δsco4677+pIBR25
Actinorhodin concentration (mg/L)
Time (day)
0
20
40
60
80
100
120
0
1
2
3
4
5
6
7
8

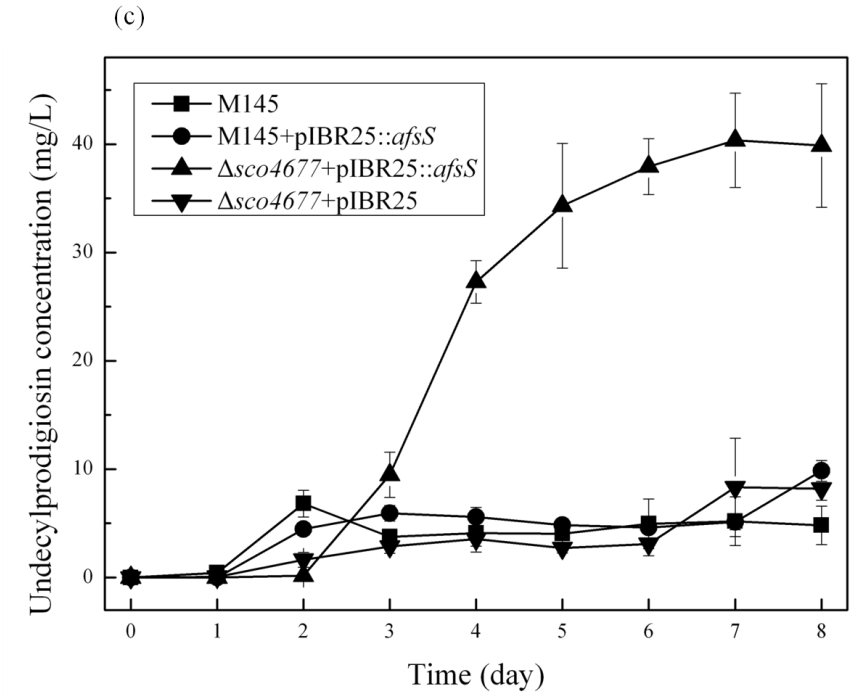


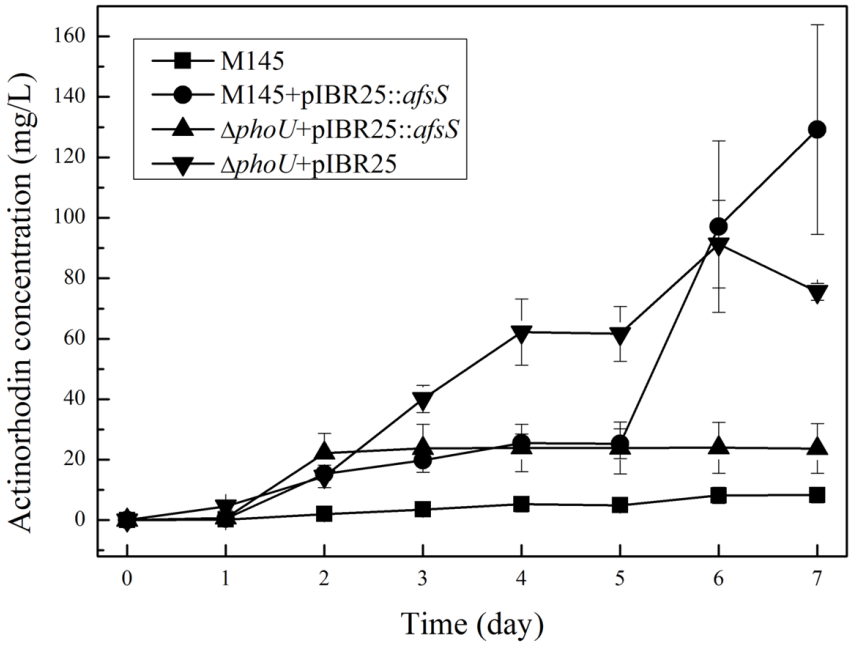
Figure 4.3 Quantification of antibiotics in M145 (■), BGafsS (M145+pIBR25::*afsS*) (●), BG4677S (Δ*sco4677*+pIBR25::*afsS*) (▲) and BG4677 (Δ*sco4677*+pIBR25) (▼) cultured in liquid $R5^-$ complex media. Intracellular ACT was dramatically increased in BG4677S and the maximum amount was 458.5 mg/L (a). Extracellular ACT was not increased compared with BGafsS (b). RED was also increased in BG4677S and its maximum amount was 40.4 mg/L (c).

4.1.3 Synergy effects of *phoU* and *afsS* double mutants in SMM without phosphate

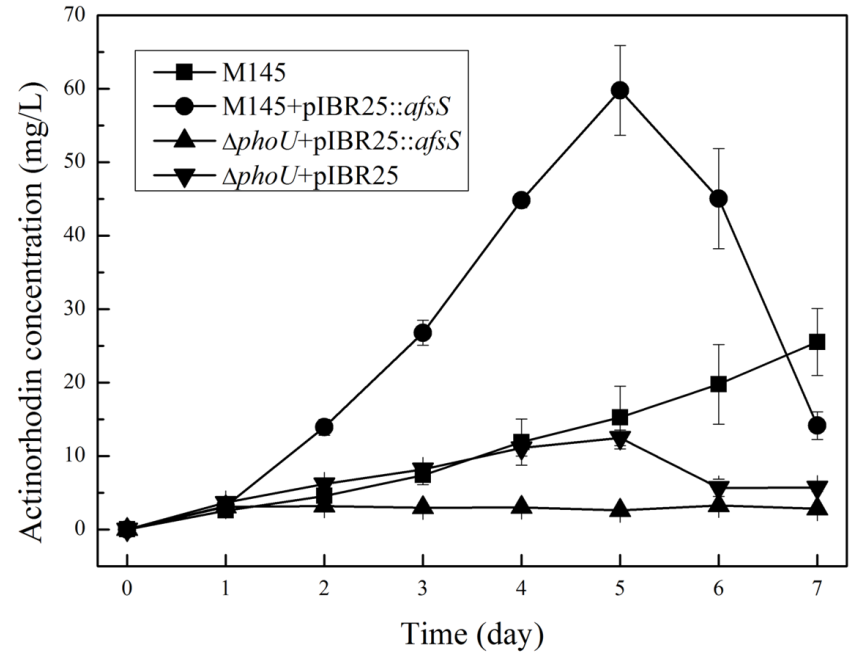
In addition to *sco4677*, we also experimentally tested another regulator, *sco4228* among the 11 up-expressed AfsS regulators. *Sco4228*, also known as *phoU*, has been considered as a regulator associated with 1) the phosphate transport system and 2) the PhoR/PhoP two-component system in which senses the phosphate level. However, the exact function of *phoU* in *Streptomyces coelicolor* is still unknown. To demonstrate the role of SCO4228 in the antibiotics production, we constructed the mutant that *phoU* was deleted or over-expressed. Since it is not clear whether *phoU* was an activator or a repressor of the antibiotics production, both *phoU* over-expressed (M145+pIBR25::*phoU*) and *phoU* deletion mutants (Δ*phoU*) were generated to assess its role in the antibiotics production. In $R5^-$ complex media, both the mutants led to no significant production of antibiotics though the production by Δ*phoU* was higher than that by M145+pIBR25::*phoU* (data not shown.) Therefore, we decided to use Δ*phoU* rather than M145+pIBR25::*phoU*, the double mutant, BG4228S (Δ*phoU*+pIBR25::*afsS*) was constructed and cultured in $R5^-$ complex media and performed the analysis of antibiotics the same as above. However, the most antibiotics enhanced mutant was BGafsS, not BG4228S. Moreover, BG4228S produced antibiotics less than wild types except intracellular ACT (Figure 4.4). So, we considered the approach to improve the production ability of BG4228S. We focused the function of the *phoU* in the cell, although it was not clear, but it would be to sense the extracellular phosphate level. Therefore, we observed the antibiotics production in solid SMM at various phosphate concentration using Δ*phoU* comparing with

M145 and M145+pIBR25::*phoU* again. All strains produced antibiotics well in SMM without phosphate condition (Figure 4.5). The production of Δ*phoU* was higher than M145+pIBR25::*phoU* as expected (Figure 4.6). Using this condition and Δ*phoU*, the quantification of antibiotics in the double mutant was performed again only considered the overproduction of antibiotics. All three antibiotics were dramatically enhanced in SMM without phosphate condition. While M145 seldom produced antibiotics in all conditions, intracellular ACT was about 149-fold increase, extracellular ACT was about 81-fold increase and RED was about 579-fold increase compared with M145, interestingly (Figure 4.7).

(a)


M145
M145+pIBR25::afsS
ΔphoU+pIBR25::afsS
ΔphoU+pIBR25
Actinorhodin concentration (mg/L)
Time (day)
0
20
40
60
80
100
120
140
160
0
1
2
3
4
5
6
7

(b)


M145
M145+pIBR25::afsS
ΔphoU+pIBR25::afsS
ΔphoU+pIBR25
Actinorhodin concentration (mg/L)
Time (day)
0
10
20
30
40
50
60
70
0
1
2
3
4
5
6
7

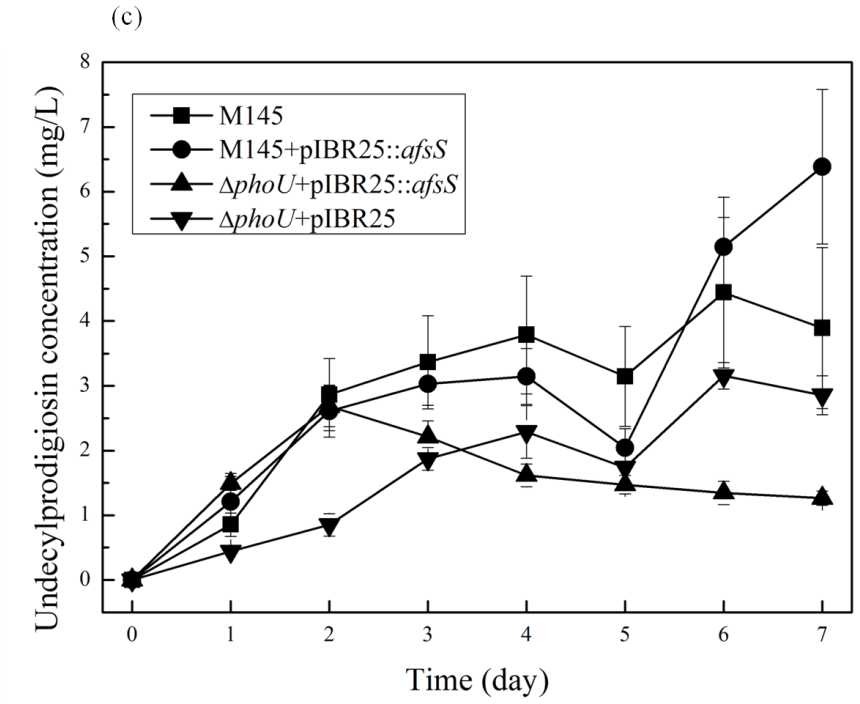


Figure 4.4 Quantification of antibiotics in M145 (■), BGafsS (M145+pIBR25::*afsS*) (●), BG4228S (Δ*phoU*+pIBR25::*afsS*) (▲) and BG4228 (Δ*phoU*+pIBR25) (▼) cultured in liquid $R5^-$ complex media. Intracellular ACT (a), extracellular ACT (b) and RED (c) were all not increased compared with other strains.

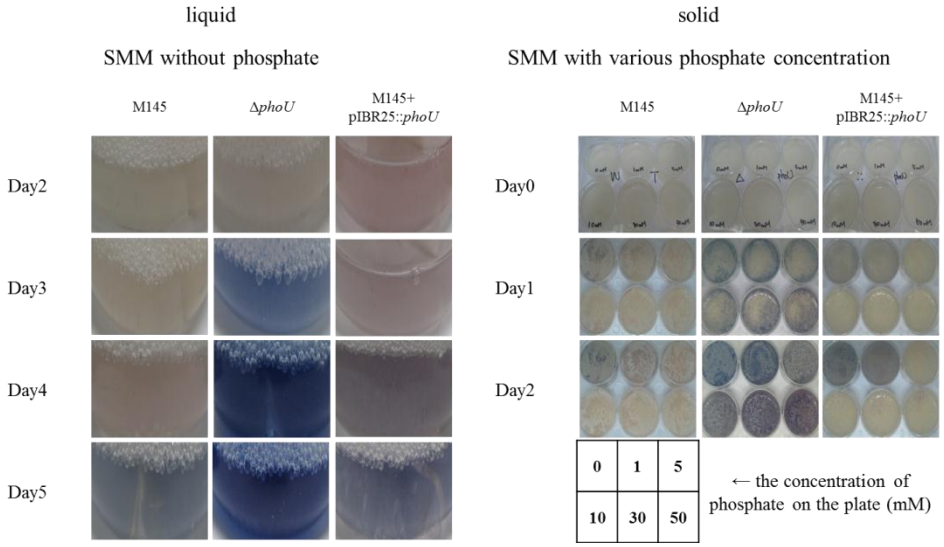
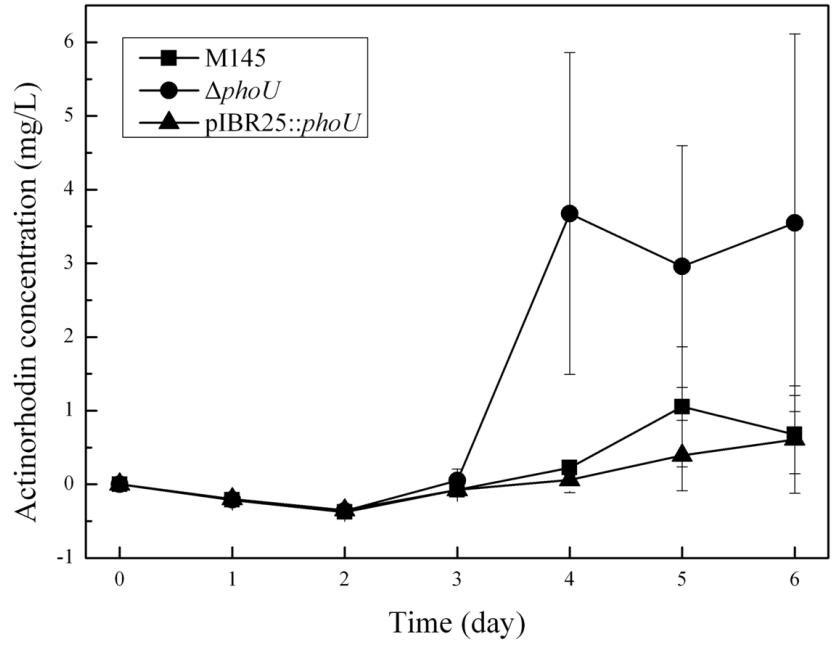
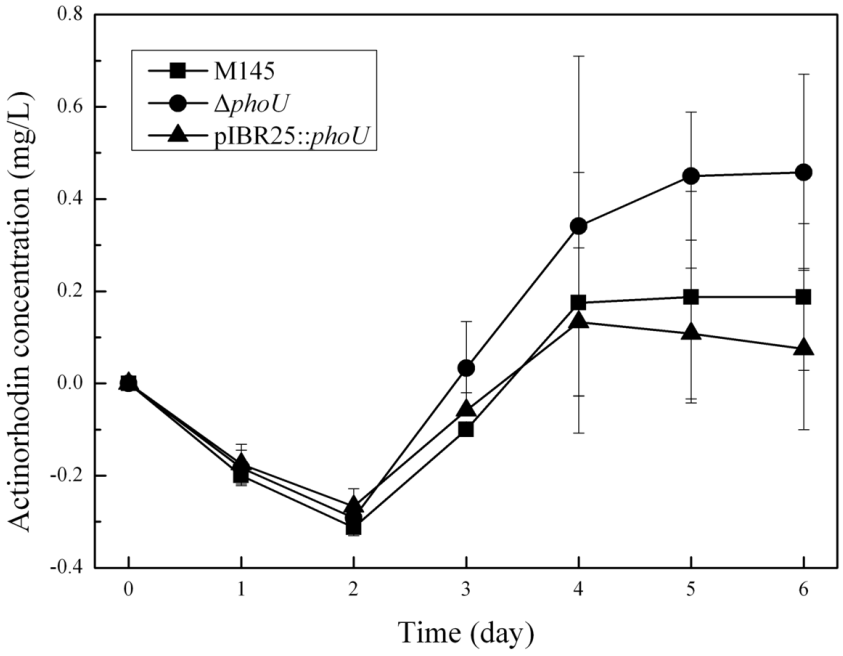


Figure 4.5 The media optimization for the antibiotics production using M145, Δ*phoU* and M145+pIBR25::*phoU*. At first, using various concentration of phosphate, proper media condition was selected and that was the condition without phosphate because all strains were produced antibiotics most. Then antibiotics production was compared in liquid SMM without phosphate.

(a)


M145
ΔphoU
pIBR25::phoU
Actinorhodin concentration (mg/L)
Time (day)
6
5
4
3
2
1
0
-1
0
1
2
3
4
5
6

(b)


M145
ΔphoU
pIBR25::phoU
Actinorhodin concentration (mg/L)
Time (day)
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
0
1
2
3
4
5
6

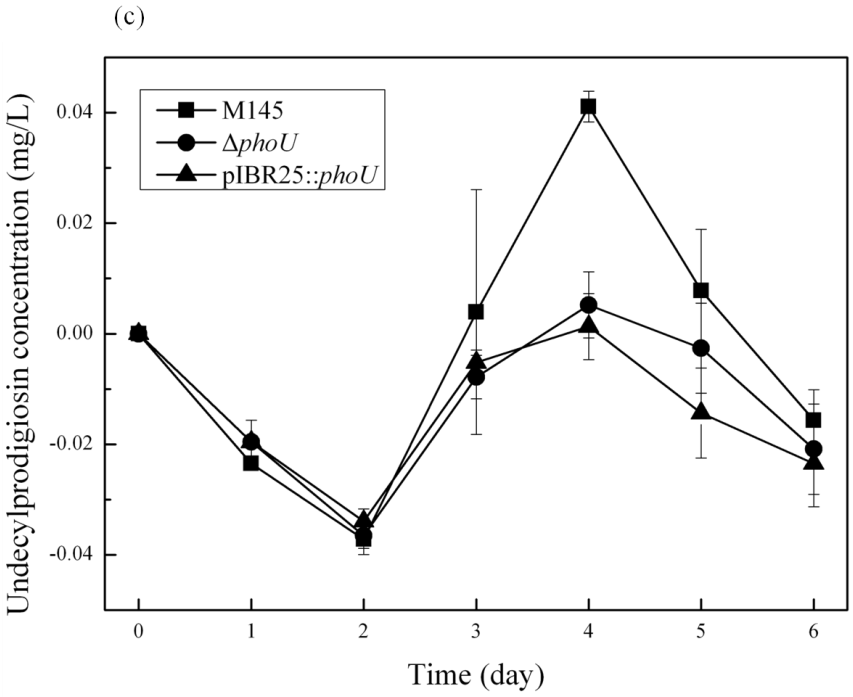
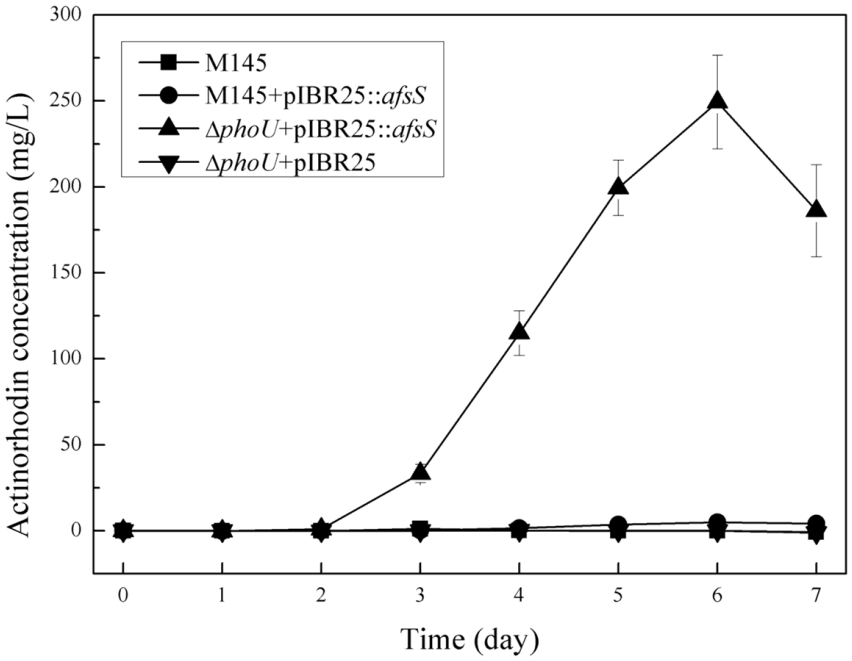
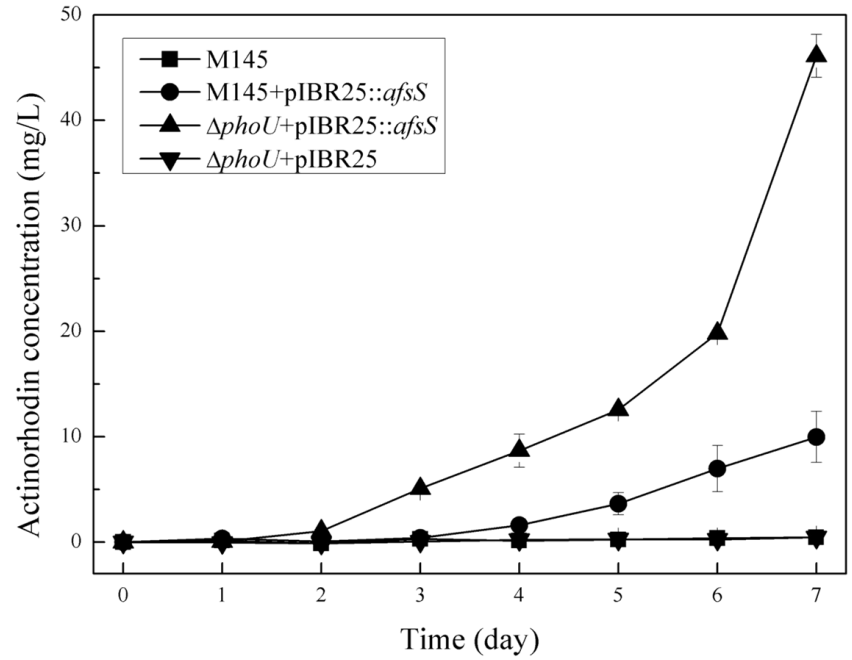


Figure 4.6 Quantification of antibiotics in M145 (■), Δ*phoU* (●) and M145+pIBR25::*phoU* (▲) cultured in liquid SMM without phosphate. Intracellular ACT (a) and extracellular ACT (b), except RED (c), were increased in Δ*phoU*.

(a)


M145
M145+pIBR25::afsS
ΔphoU+pIBR25::afsS
ΔphoU+pIBR25
Actinorhodin concentration (mg/L)
Time (day)
0
50
100
150
200
250
300
0
1
2
3
4
5
6
7

(b)


M145
M145+pIBR25::afsS
ΔphoU+pIBR25::afsS
ΔphoU+pIBR25
Actinorhodin concentration (mg/L)
Time (day)
0
10
20
30
40
50
0
1
2
3
4
5
6
7

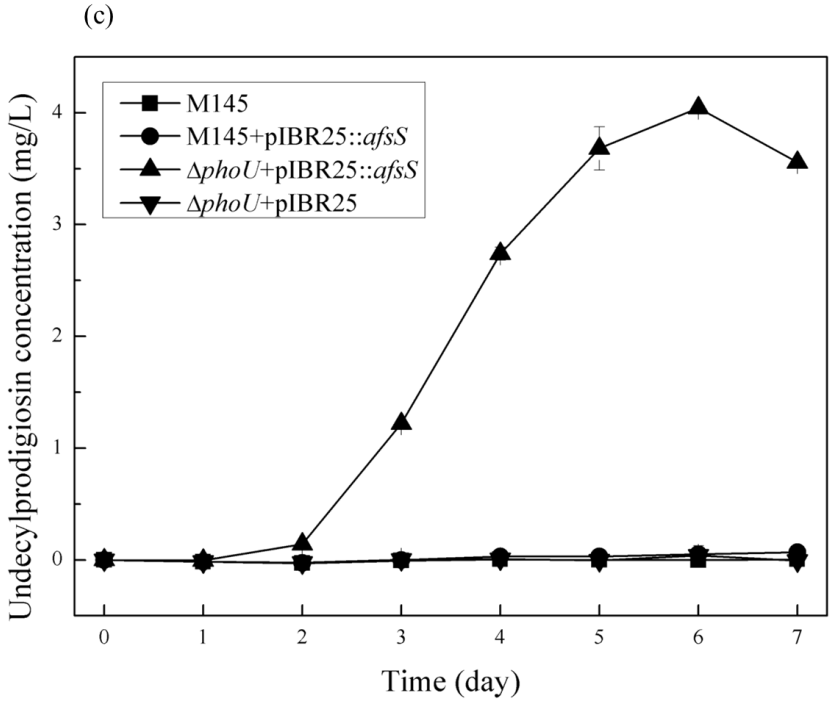


Figure 4.7 Quantification of antibiotics in M145 (■), BGafsS (M145+pIBR25::*afsS*) (●), BG4228S (Δ*phoU*+pIBR25::*afsS*) (▲) and BG4228 (Δ*phoU*+pIBR25) (▼) cultured in liquid SMM media without phosphate. Intracellular ACT in BG4228S reached at 249.4 mg/L (a). Extracellular ACT was also increased at maximum 46.1 mg/L (b). RED was increased in BG4228S and its maximum amount was 4 mg/L (c). Interestingly, M145 seldom produced antibiotics in all conditions in contrast BG4228S produced well.

4.2 Conclusion

For increasing antibiotic production, we performed the analysis of microarray data and selected target regulators. Even though about 700 regulators are annotated in *Streptomyces coelicolor*, most regulators are still known a little. In this status, we suggested the approach to enhanced antibiotics by using regulators. Regulators are constructed multi-layered networks (Baumbach, 2007) and pathway-specific regulators like ActII-ORF4, RedD and RedZ are controlled by other regulators. One of those regulators is AfsS, which is an activator of antibiotic production. The information about regulators was rarely revealed but by using microarray data we could select the regulators which showed transcriptionally independent expression patterns with *afsS*. The potential candidates were belonged in up-regulated gene cluster because they were probably involved in important cellular events such as secondary metabolism and morphological development.

In the case of the double mutant that was *afsS* over-expressed and *sco4677* deleted, antibiotics were enhanced expectedly. We assumed that these phenomena were occurred because of the synergy effects, which means that *afsS* and *sco4677* have transcriptionally independent regulatory network in antibiotics biosynthesis and they do not interfere in their own function during growth. In contrast, we could observe the SEM image of the double mutant that BG4677S delayed the formation of spores and morphological development (Figure 4.8). The known function of *sco4677* is the antagonistic regulator of sigma factor F, which means that Δ*sco4677* would be forced into accelerating sporulation.

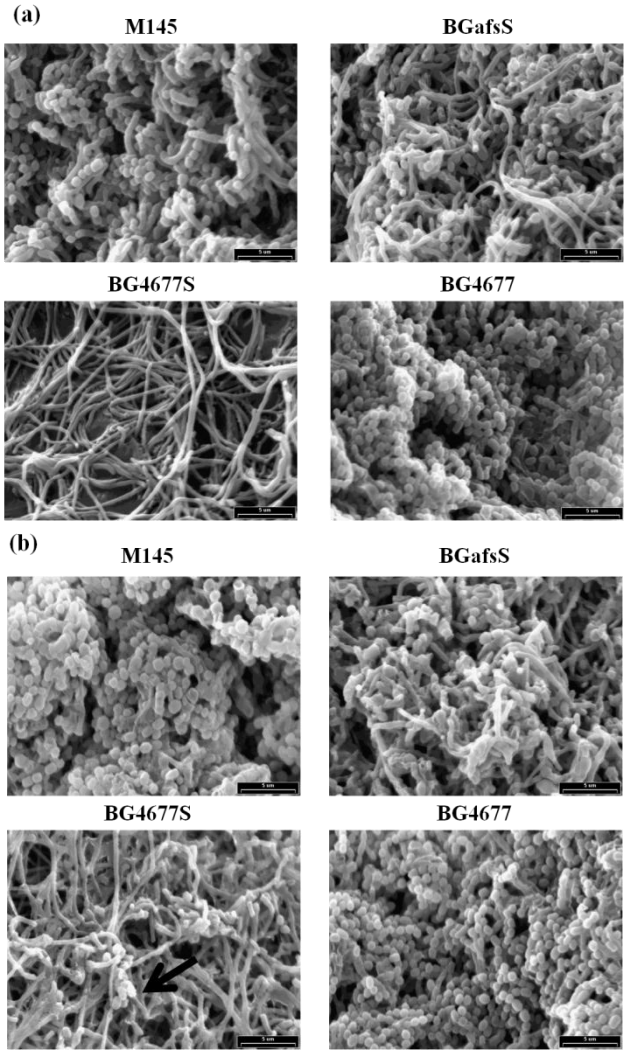


Figure 4.8 SEM images of M145, BGafsS (M145+pIBR25::*afsS*), BG4677S (Δ*sco4677*+pIBR25::*afsS*) and BG4677 (Δ*sco4677*+pIBR25). BG4677S could not sporulate until 5-day culture on the $R5^-$ complex media plate (a). Late sporulation of BG4677S was observed in 8-day culture on the $R5^-$ complex media plate (b).

In SEM data, BG4677 had more spores than wild type, but BG4677S was quite delayed the morphological development. So, we supposed that BG4677S could start to overproduce antibiotics from the earlier developmental stage than the wild type rather than the stationary phase because of the mutation.

For more enhancing antibiotics production, other suggestion is the point of the extracellular export. In the quantitative analysis, intracellular ACT was enhanced enough, but extracellular ACT was similar with *afsS* over-expressed mutant as shown in Figure 4.3. Several studies showed that antibiotics efflux pumps such as AvtAB for avermectin (Qiu et al., 2011) and DrrAB for doxorubicin (Guilfoile & Hutchinson, 1991) were related with the resistance against antibiotics and the secretion of antibiotics. In *Streptomyces coelicolor*, efflux pump system of ACT has already been reported that ActA as an efflux pump and ActR as a repressor to control the transcription of ActA (Tahlan et al., 2007). Therefore, if the export of ACT is increased by control the ActA or ActR, total production of ACT would be also enhanced because of the increase of the resistance against cytotoxicity.

Although the role of *phoU* is not fully revealed yet, we presumed this phenomenon on BG4228S came from the relation with phosphate metabolism. It has already known that PhoP and AfsR are possible to cross-regulate the transcription of *afsS* by binding the promoter region. If *phoU* is deleted, the activation of PhoR/P signal transduction depending on the phosphate level will be weakened because PhoU regulates the phosphate transport with PstSCAB. And the repression by phosphorylated PhoP will be also weakening on *afsS*. Thereby *afsS* will be most strongly activated in the case of the double mutant in SMM without phosphate.

It requires more studies to understand what happened on the BG4677S and BG4228S, but their remarkable capacity to produce antibiotics has been shown enough by our studies. In the situation of the lack of the information about the transcriptional regulatory network, we could develop the efficacious method using the microarray data for overproducing antibiotics. Recently, Murakami and coworkers reported that antibiotics multiples by the targeted amplification of the antibiotics biosynthetic gene clusters (Murakami et al., 2011). This approach is quite direct and effective, but affects only target gene clusters. However, our approach influences globally through the transcriptional regulatory network. For the maximum production of antibiotics, we suppose that both approaches apply in the cell at once, more enhancement of productivity will be occurred. Moreover, media optimization and other approaches mentioned at first help to overproduce antibiotics as well.

Chapter V

Analysis of transcriptional regulatory network of *ndgR* deletion mutant using time-course microarray

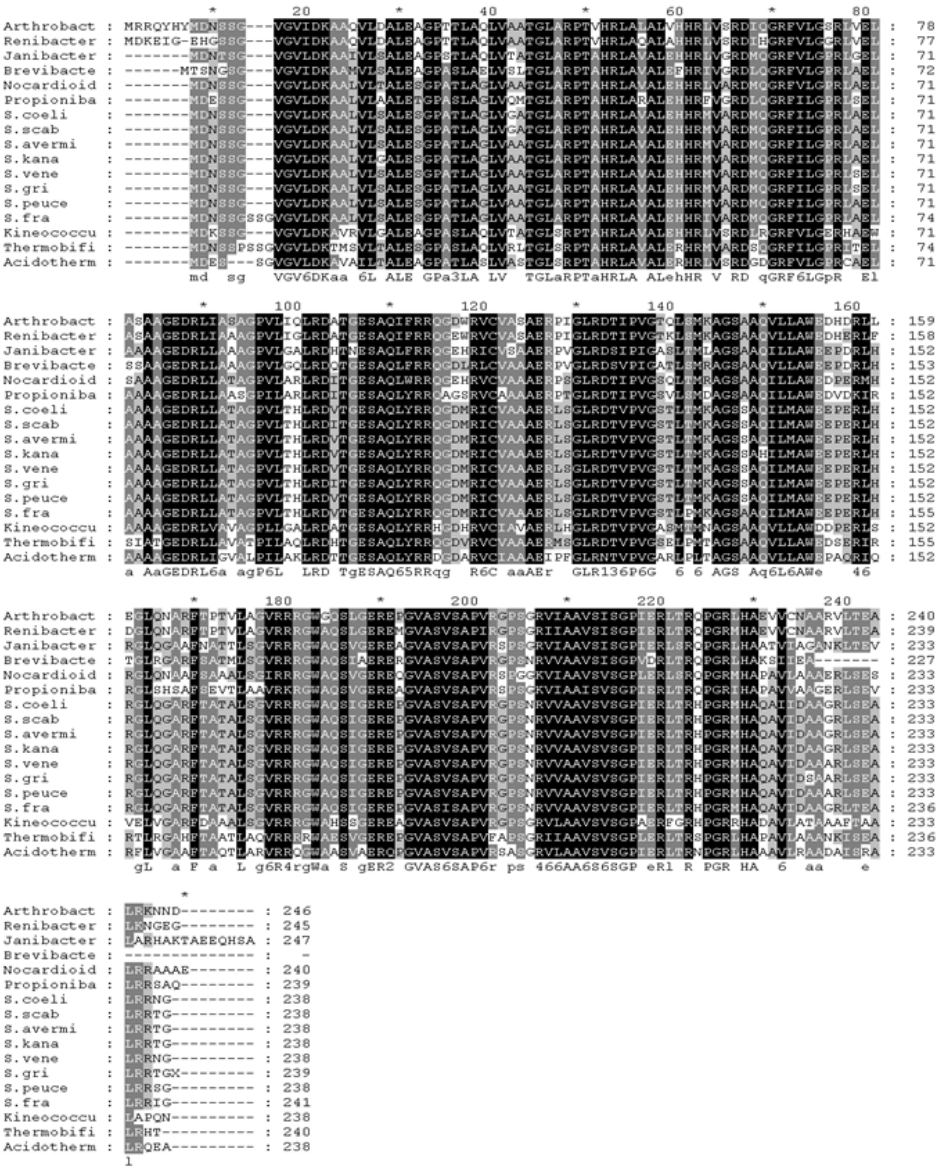
5.1 NdgR, an IclR-like regulator in *Streptomyces coelicolor*

Previously, a new regulator, NdgR (Yang et al., 2009) was identified by DNA affinity capture assay (DACA) and mass spectrometric analysis. NdgR was found in five promoter sequences of doxorubicin biosynthetic gene cluster except *dnrN* promoter in *Streptomyces peucetius* in order to find new transcriptional regulators related to the biosynthetic pathways of doxorubicin production. Moreover, by sequence alignment, *ndgR* was shown with high homologous sequence in *Streptomyces*, especially 99% between *S. coelicolor* and *S. avermitilis*. Furthermore, 5'-UTR sequences of *ndgR* in *Streptomyces* were almost identical (Figure 5.1). Therefore, we assumed that the cellular function of NdgR should be highly conserved in *Streptomyces*.

To find the function of *ndgR*, we made a *ndgR* deletion mutant, BG11 and previously performed two-dimensional gel electrophoresis and DNA microarray experiments in the complex $R5^-$ liquid media, and the antibiotic production mapping method using 96-well plate with various carbon and nitrogen sources. From those results, NdgR was identified to involve with nitrogen source dependent growth, antibiotics production, quorum sensing and expression of chaperonins as well.

To elucidate the role of NdgR in detail, we performed the screening of the media component such as carbon and nitrogen sources with BG11, the best experiment to show distinct differences between the wild type and BG11. Then, the condition, which was solid minimal media in N-acetyl glucosamine as a carbon source, and L-asparagine as a nitrogen source were selected for further study. To understand the global role of NdgR, we performed time-course microarray experiments.

(a)



(b)

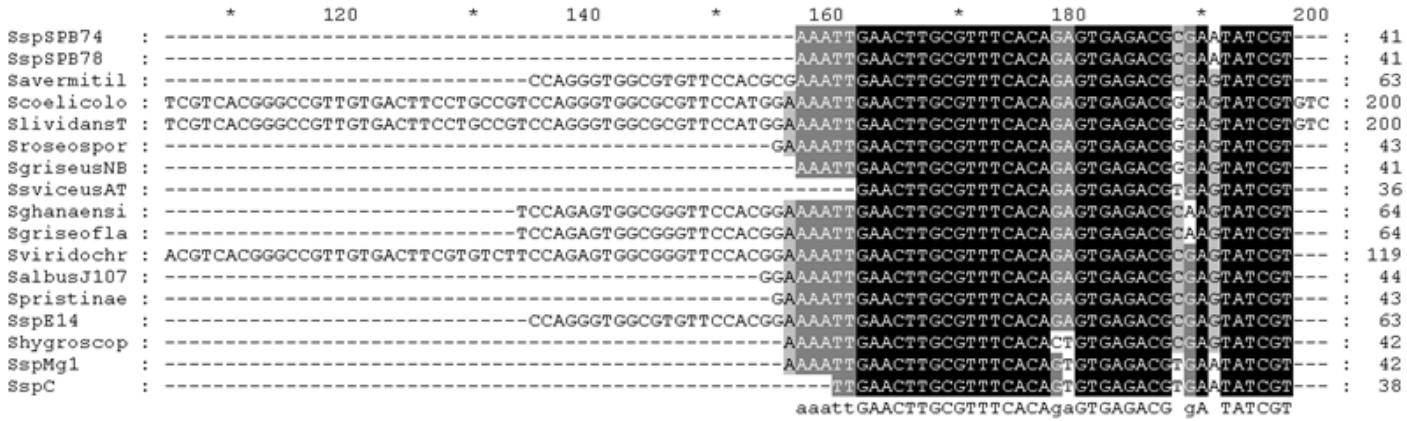


Figure 5.1 *NdgR* is highly conserved in *Streptomyces*. NdgR with more than 98% similarity in *Streptomyces* (a), and 5' UTR of *ndgR* was also conserved in *Streptomyces* (b).

5.2 Time-course microarray experiments of *ndgR* deletion mutants

5.2.1 RNA sample preparation and Array experiments

Depending on carbon and nitrogen sources, BG11 showed defferent phenotypes. Among them, we chose N-acetyl glucosamine as a carbon source and L-asparagine as a nitrogen source for the solid minimal media (MM GlcNAc/ASN) (shown in Figure 5.2). In MM GlcNAc/ASN, BG11 showed slow growth and early production of actinorhodin (ACT) even from 15 h after inoculation, compared to the wild type. Undecylprodigiosin (RED) was not observed in BG11. On the other hand, there were little physiological differences in $R5^-$ complex media culture between wild type and BG11 (Figure 5.3). For the microarray experiment, we collected the wild type cells and BG11 using cellophane covered solid MM GlcNAc/ASN at 36, 48, 60 and 72 h. Detail experimental procedures are mentioned in section 2.6 DNA microarray of Chapter II.

5.2.2 Data analysis

The raw data obtained from the microarray experiments were normalized as mentions in section 2.6 DNA microarray of Chapter II. By clustering analysis, we could show ten major patterns of mRNA expression of BG11 compared to the wild type. The ten major expression patterns are shown in Figure 5.4. Red means up-regulated patterns in BG11 compared to the wild type and green means down-regulated patterns in BG11. 549 genes are up-regulated and contains major cluster which up-regulated the best at 36 h and were preserved until 72 h. The second major cluster includes about 338 genes and highly down-regulated patterns in all the time points. The other

clusters also shows distinct expression patterns such as up to down, down to up and so on.

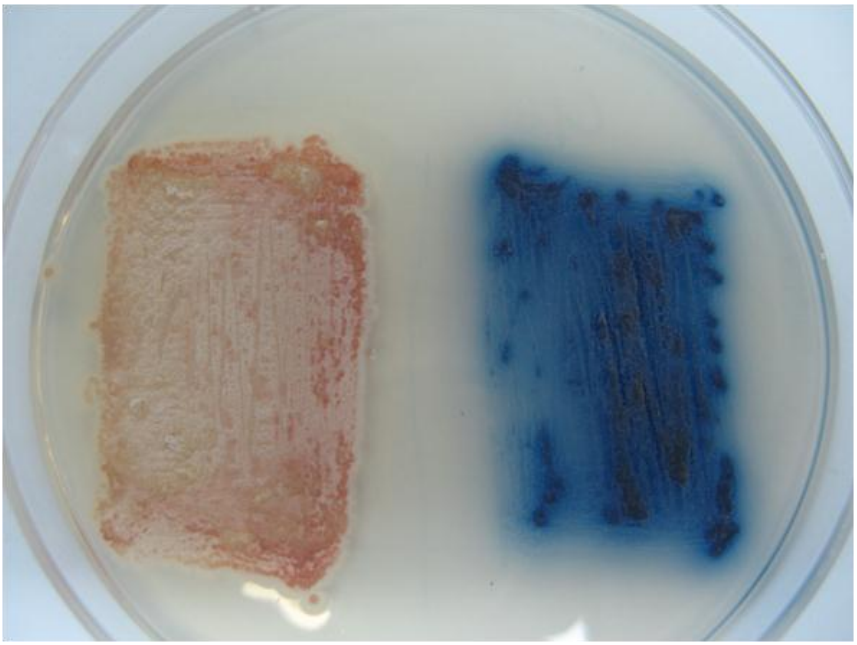


Figure 5.2 Different phenotype between wild type (left) and BG11 (right) grown in solid MM GlcNAc/ASN.

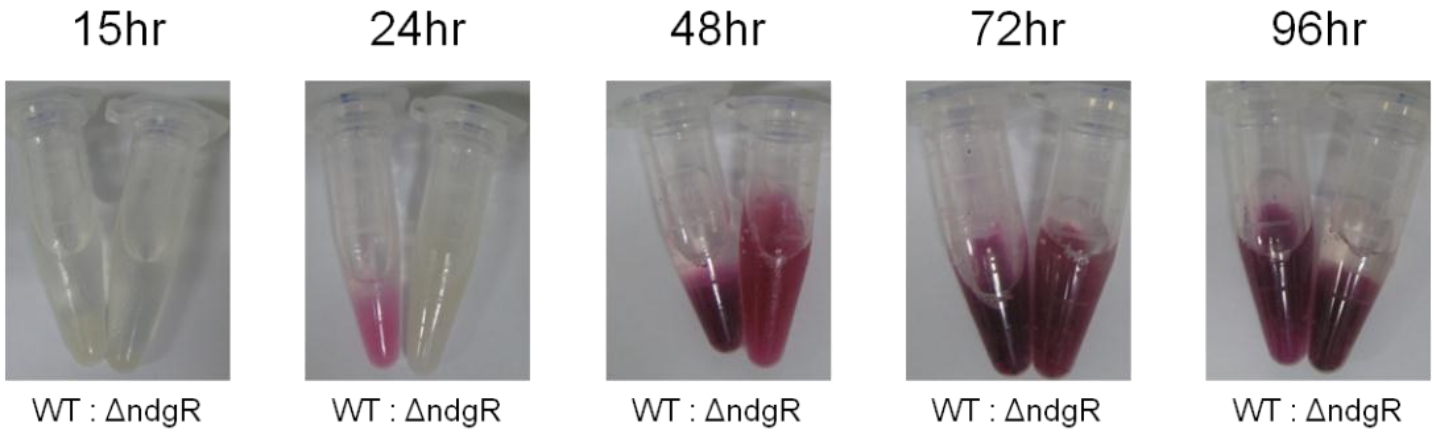


Figure 5.3 Observation of wild type and BG11 (Δ*ndgR*) grown in $R5^-$ liquid culture. They are quite similar except the antibiotics producing time. The Wild type produces antibiotics earlier than BG11.

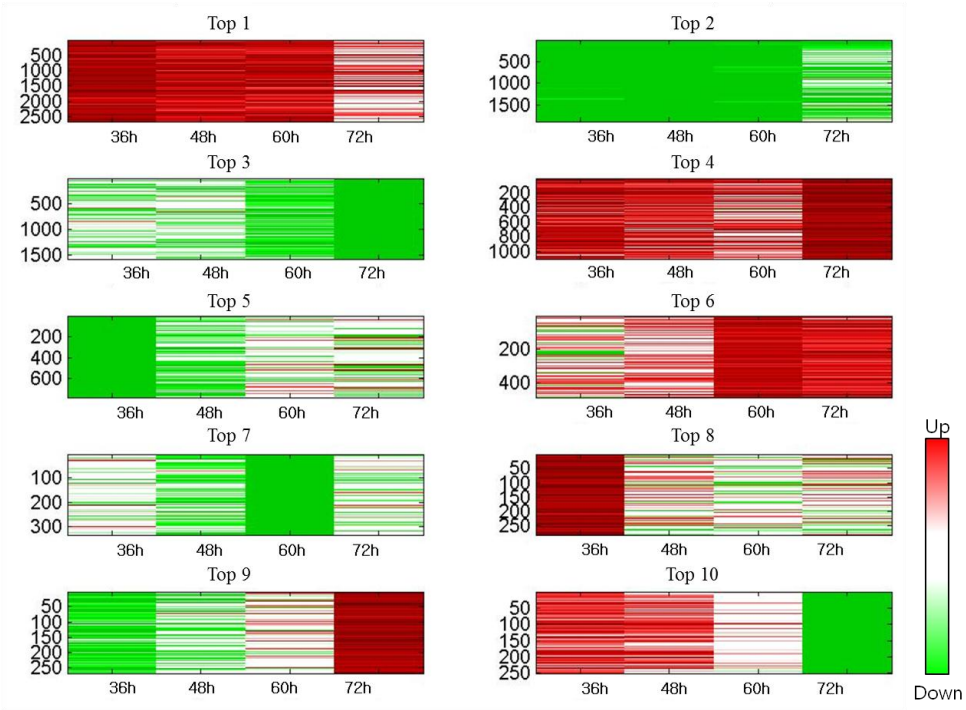


Figure 5.4 Major mRNA expression patterns categorized by clustering analysis. Red indicates up-regulated genes in BG11 compared to wild types, and green indicates down-regulated genes in BG11 compared to wild types. X-axis: time, Y-axis: number of genes.

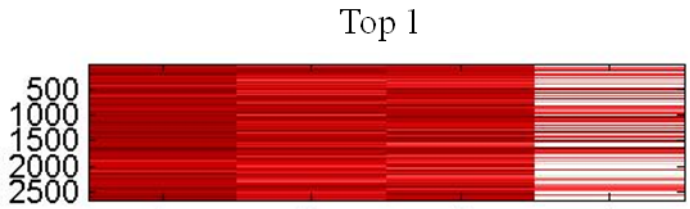
At first, using clustered gene lists, we performed gene ontology analysis. Gene Ontology (GO) is like collection of controlled vocabularies depending on the biology of a gene product in any organism. Therefore, with analysis of the GO, we can assume BG11 has the global role because GO analysis showed species independent and consistent descriptions of gene products.

Genes belong to the top 1 cluster were analyzed according to their biological process as a DNA integration, DNA metabolic process, carbohydrate transport and so on. In DNA metabolic process, there were DNA glycosylase, endonuclease V and insertion element. However, 487 genes among 549 genes were not included in the analyzed list (Figure 5.5).

Genes in the top 2 cluster were also analyzed according to their biological process as a homeostatic process, cellular homeostasis, cell redox homeostasis and so on. Also, 308 genes among 326 genes were not included in the list (Figure 5.6).

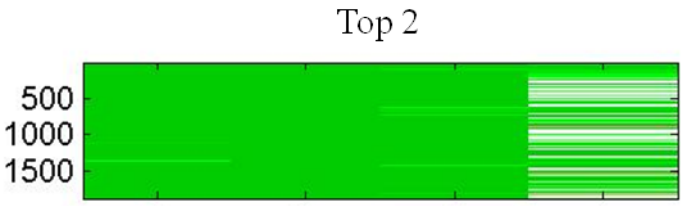
Many genes were missed for the analysis, because those in *Streptomyces coelicolor* remained as putative functions. With such limited database, analyzing *S. coelicolor* gene ontology is difficult.

Next, we used the normalized data of the interest and analyzed it directly to reveal their metabolic pathways. Then we presumed the function of *ndgR* according to the fold changes compared to wild type. Table 5.1 shows the metabolic pathways related with gene clusters. Some genes are related with the same metabolic pathways, and we assumed that there are more precise control of metabolic flux to survive without *ndgR* in those metabolic pathways.



Biological function	**Number of genes**
DNA integration	9
DNA metabolic process	23
nucleobase, nucleoside, nucleotide and nucleic acid transport	3
carbohydrate transport	8
glycerol metabolic process	5
alditol metabolic process	5
transposition, DNA-mediated	6
transposition	6
carboxylic acid transport	5
organic acid transport	5

Figure 5.5 Gene ontology analysis using the genes in top 1 cluster. Only 52 genes except the overlapping ones among 549 genes are included in the list.



Biological function	Number of genes
homeostatic process	8
cellular homeostasis	7
cell redox homeostasis	6
iron ion transport	3
cell adhesion	3
biological adhesion	3
DNA replication	5

Figure 5.6 Gene ontology analysis using the genes in top 2 cluster. Only 18 genes except the overlapping ones among 326 genes are included in the list.

Table 5.1 Metabolic pathways that were up-regulated in top 1 cluster and down-regulated in top 2.

Up-regulated pathway in Top 1 cluster
ABC transporters
chlorocyclohexane and chlorobenzene degradation
naphthalene degradation
pyruvate metabolism
amino sugar and nucleotide sugar metabolism
biosynthesis of type II polyketide backbone
butanoate metabolism
cyanoamino acid metabolism
fatty acid metabolism
glycerolipid metabolism
glycerophospholipid metabolism
glycolysis/gluconeogenesis
glyoxylate and dicarboxylate metabolism
histidine metabolism
oxidative phosphorylation
phenylalanine metabolism
starch and sucrose metabolism
TCA cycle
terpenoid backbone biosynthesis
tyrosine metabolism
valine, leucine and isoleucine biosynthesis

Down-regulated pathway in Top 2 cluster
ABC transporters
chlorocyclohexane and chlorobenzene degradation
naphthalene degradation
pyruvate metabolism
cysteine and methionine metabolism
fatty acid biosynthesis
geraniol degradation
methane metabolism
pantothenate and CoA biosynthesis
phosphotransferase system(PTS)
porphyrin and chlorophyll metabolism
ribosome

5.2.3 Up-regulated genes in BG11

Previously, we showed NdgR was nitrogen dependent growth and antibiotics production regulator. In minimal media contained glucose as a carbon source and ILE, LEU, ASN, VAL, MET and glutamine as a nitrogen source, the differences in the antibiotics production was observed (data not shown). And *areB* mutant in *Streptomyces clavuligerus* showed the nutritional impairment in minimal media especially with ILE, LEU, VAL and fatty acids. In addition, the location of *ndgR* was upstream of *leuCD* involved in ILE, LEU and VAL biosynthesis.

In microarray data, we confirmed previous data again. The pathway of ILE, LEU and VAL biosynthesis was activated in BG11 except *leuCD* as previously reported. And, *glnA*, a glutamine synthetase I related with the glutamate metabolism was also activated in BG11. (shown in Figure 5.7 and Figure 5.8).

Interestingly, we could identify that *ndgR* was involved the glutamate transport system as well as the glutamate metabolism. We found the up-regulation of ABC transport system such as glutamate transport system and branched-chain amino acid transport system and down-regulation of ABC transport system such as phosphate transport system and methionine transport system in BG11 (Figure 5.9).

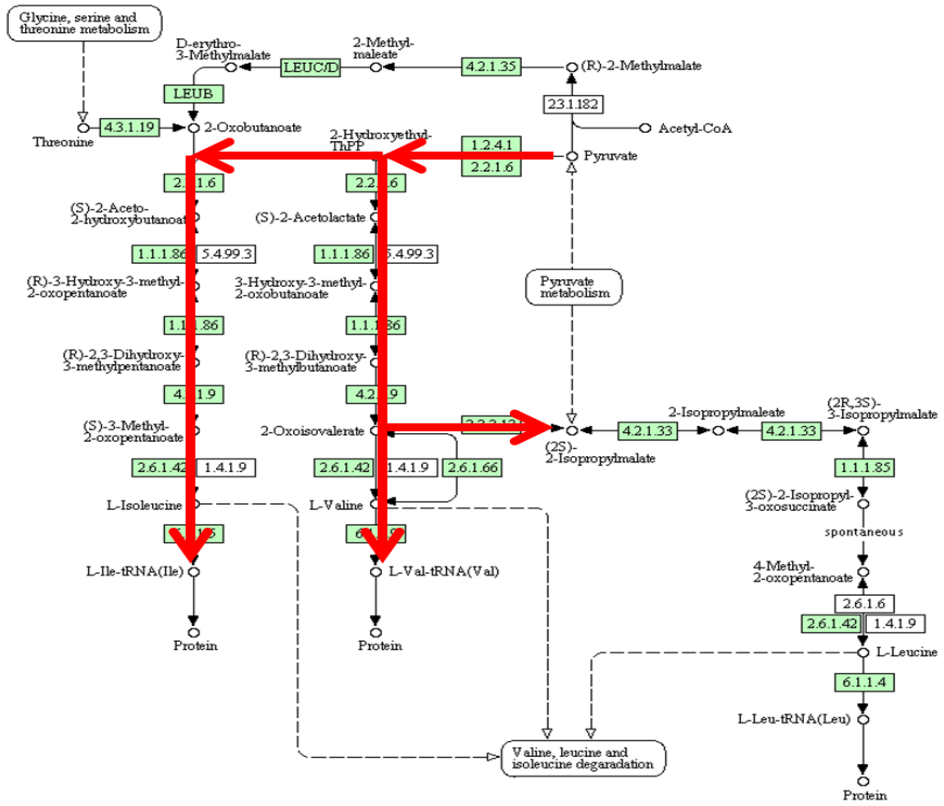


Figure 5.7 Activation of Val, Leu and Ile biosynthesis in BG11. Most genes are affected by NdgR. Red arrows indicate up-regulated pathways in BG11.

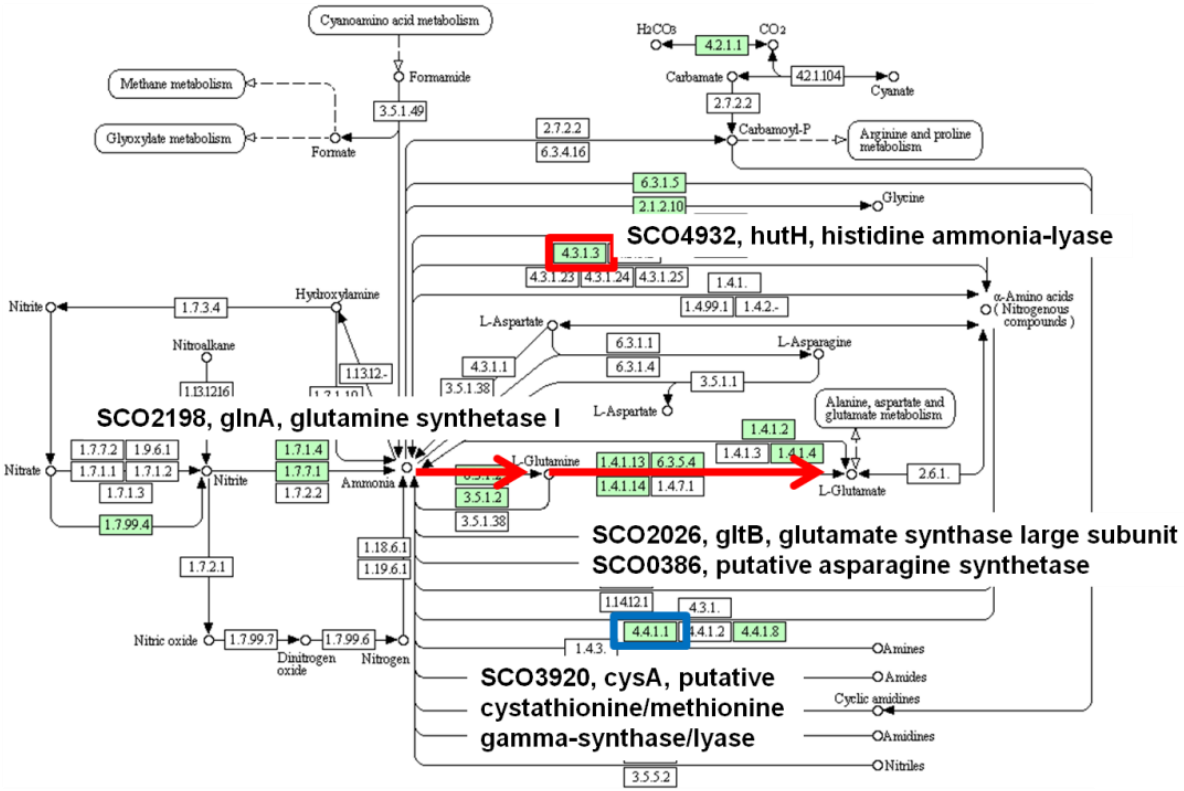


Figure 5.8 NdgR affects nitrogen metabolism. Red means up-regulated genes and pathways in BG11. Blue means down-regulated genes in BG11.

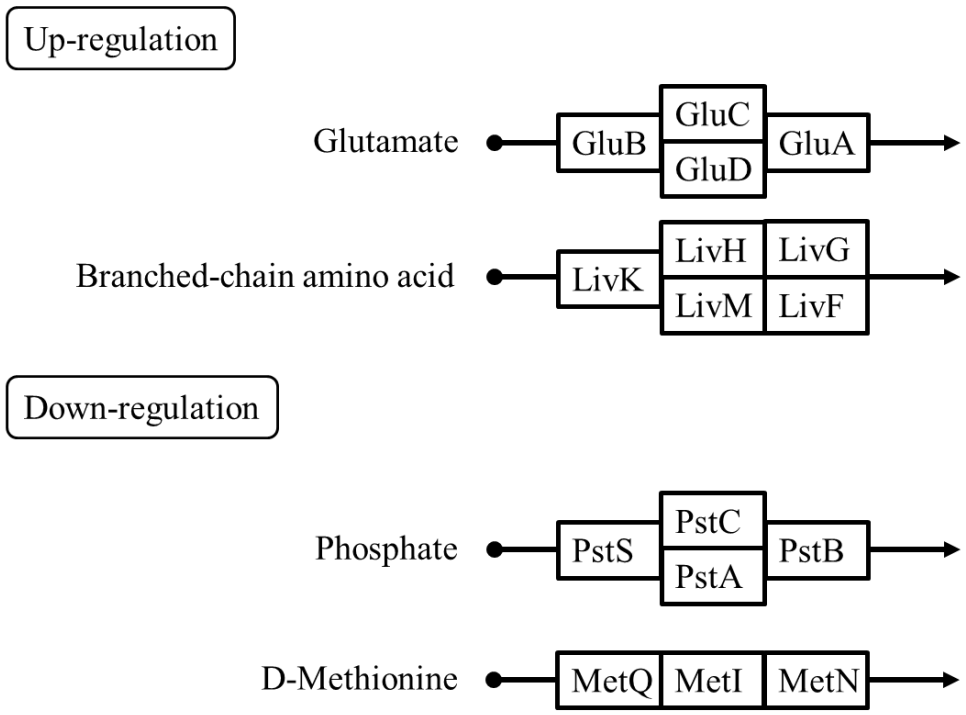


Figure 5.9 NdgR affects various ABC transport systems. Glutamate transport and branched-chain amino acid transport were up-regulated in BG11, and phosphate transport and methionine transport were down-regulated in BG11.

Glutamate transport system was consisted of four genes, *gluABCD*. Genes encode glutamate uptake system ATP-binding protein (*gluA*), glutamate binding protein (*gluB*), and glutamate permease (*gluC* and *gluD*). And four genes were two to four folds up-regulated in BG11 (Figure 5.10). We performed EMSA whether NdgR was bound the promoter region of *gluABCD* to regulate the expression or not. As shown in Figure 5.11, NdgR was bound the promoter region of glutamate transport system operon and released when the non-labeled probes were added as a competitor.

Although EMSA was *in vitro* assay to confirm the binding of specific promoters with the interested protein, we supposed that glutamate transport system should be a target of NdgR to regulate glutamate uptake.

Glycerol utilization in *Streptomyces coelicolor* is determined by the glycerol operon consisted of three genes. In the upstream of glycerol operon, gylR as a negative autoregulator was located (Hindle & Smith, 1994). Glycerol operon was controlled by the glucose according to the carbon catabolite repression as well as the specific regulator, gylR. In this study, glycerol operon was significantly up-regulated and the fold change was more than eight fold in BG11, even the own regulator, gylR was not down-regulated in BG11. By EMSA, we confirmed that NdgR was bound in the promoter region of glycerol operon, which was in between gylR and glycerol operon (Figure 5.12 and Figure 5.13).

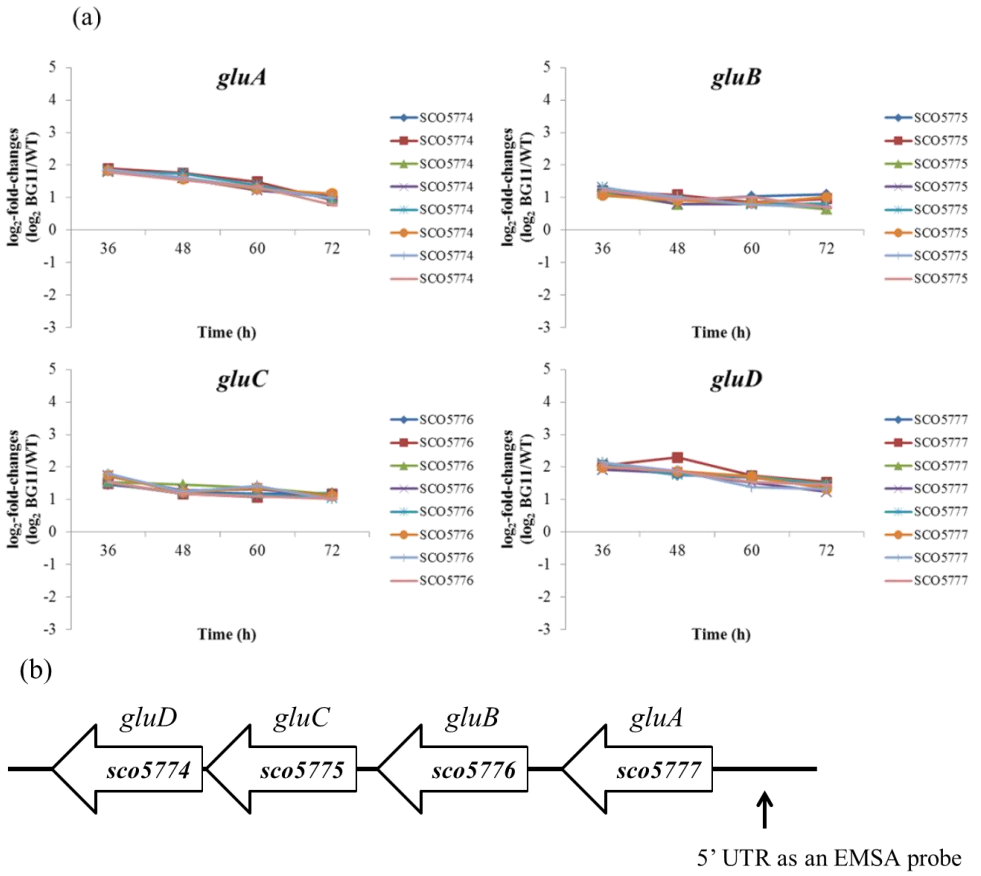


Figure 5.10 Expression pattern of glutamate transport system. Eight probes per one gene showed same expression patterns (a). All probes were up-regulated in BG11 compared to wild types. X-axis: time points, Y-axis: log_2-fold changes. Locations of genes and EMSA probe (b).

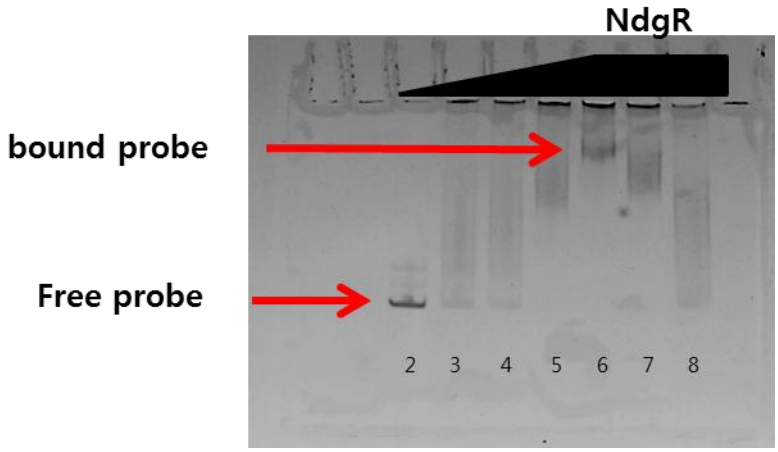


Figure 5.11 EMSA using the promoter sequence of glutamate transport system operon. Lane 2, only probes (50 ng/μL) ; lane 3, probes and NdgR (1 μg); lane 4, probes and NdgR (2 μg); lane 5, probes and NdgR (4 μg); lane 6, probes and NdgR (10 μg); lane 7, probes, 50 ng of non-labeled probes as competitors, and NdgR (10 μg); lane 8, probes, 200 ng of non-labeled probes as competitors, and NdgR (10 μg).

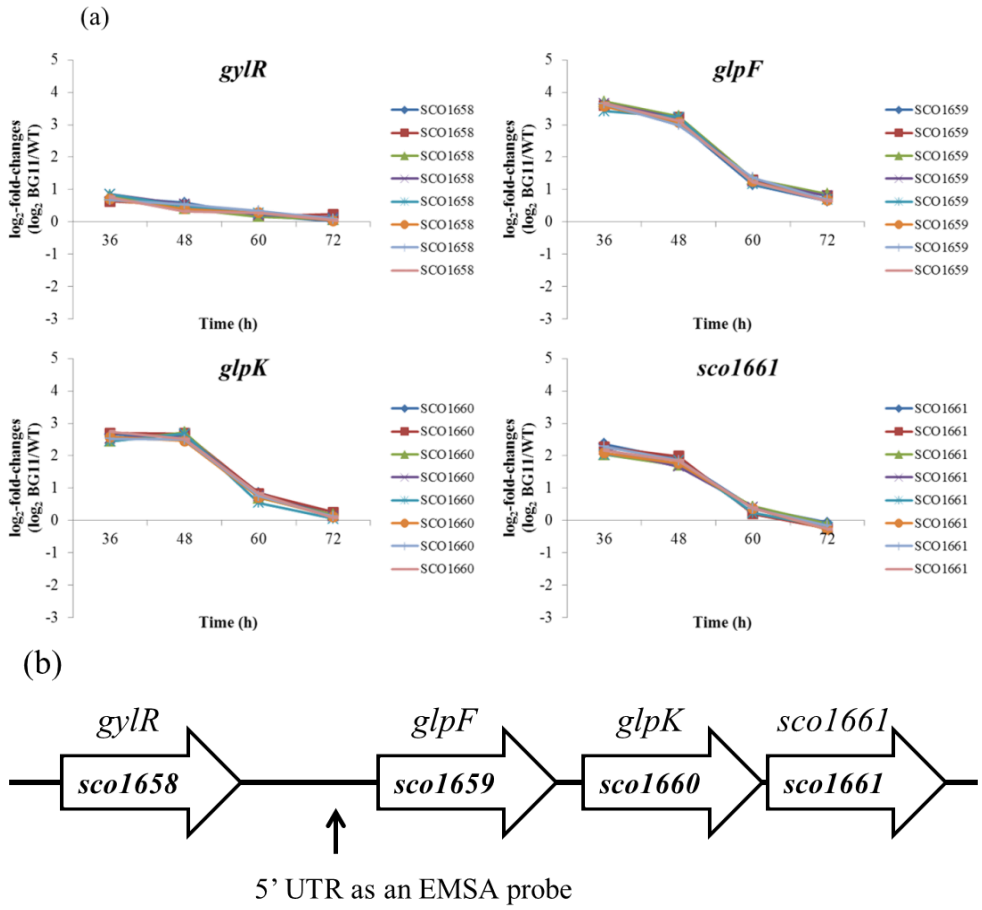


Figure 5.12 Expression pattern of glycerol operon. Eight probes per one gene showed same expression patterns (a). All probes were up-regulated in BG11 compared to wild types. X-axis: time points, Y-axis: log_2-fold changes. Locations of genes and EMSA probe (b).

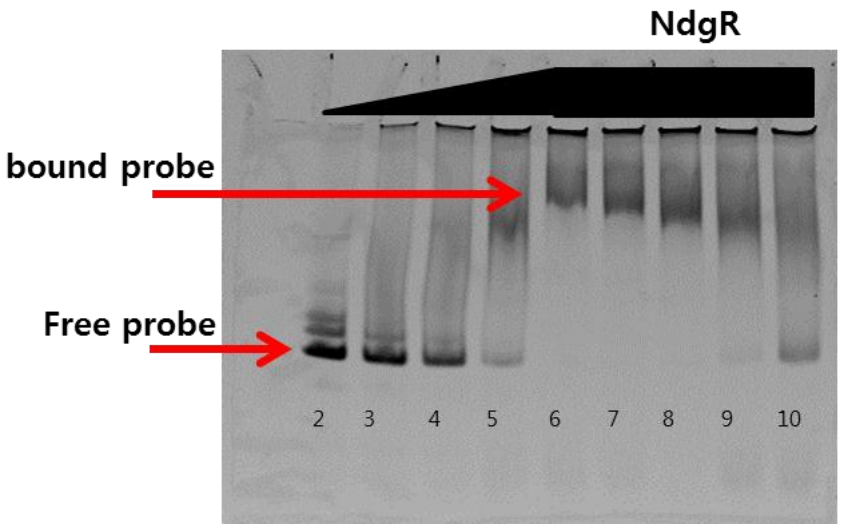


Figure 5.13 EMSA using the promoter sequence of glycerol operon. Lane 2, only probes (97 ng/μL) ; lane 3, probes and NdgR (1.25 μg); lane 4, probes and NdgR (2.5 μg); lane 5, probes and NdgR (5 μg); lane 6, probes and NdgR (12.5 μg); lane 7, probes, 100 ng of sheared salmon sperm DNA as competitors, and NdgR (12.5 μg); lane 8, probes, 500 ng of sheared salmon sperm DNA as competitors, and NdgR (12.5 μg); lane 9, probes, 97 ng of non-labeled probes as competitors, and NdgR (12.5 μg); lane 10, probes, 485 ng of non-labeled probes as competitors, and NdgR (12.5 μg).

5.2.4 Down-regulated genes in BG11

In *Streptomyces coelicolor*, several carbon uptake systems were previously reported. As shown in Figure 5.14, glycerol is facilitated and glucose is transported by the symport system. Some carbohydrates uses ABC transport system and other carbohydrates such as fructose are uptaken by phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS). In our study, we used N-acetyl glucosamine (GlcNAc) as a carbon source. There are reports on GlcNAc that it can be transported by PTS for utilization. Interestingly, in the microarray data, several genes were down-regulated in BG11. Among them, IIA^{Crr} (*sco1390*, PTS system sugar phosphotransferase component IIA, Crr) and EI (*ptsI*, *sco1391*, phosphoenolpyruvate-protein phosphotransferase) were about two fold down-regulated in BG11 (Figure 5.15). Moreover, known genes, which relate with the GlcNAc uptake system reported by Nothaft et al (Nothaft et al., 2010), were all down-regulated in BG11 (Figure 5.16 and Figure 5.17). IIA^{Crr} and EI are essential proteins to uptake carbohydrate that they have the function for phosphorylation of carbohydrates to make the suitable forms to transport into the cell. Down-regulation of IIA^{Crr} and EI means that all carbohydrates uptaken by PTS were not transported into cells because of the lack of phosphorylation. Therefore, we tested the binding of NdgR to the promoter region of IIA^{Crr} and EI *in vitro* by EMSA. As expected, the band shift was observed, and NdgR was bound to the promoter region. In this study, we firstly found that NdgR is related with PTS carbon uptake system (Figure 5.18).

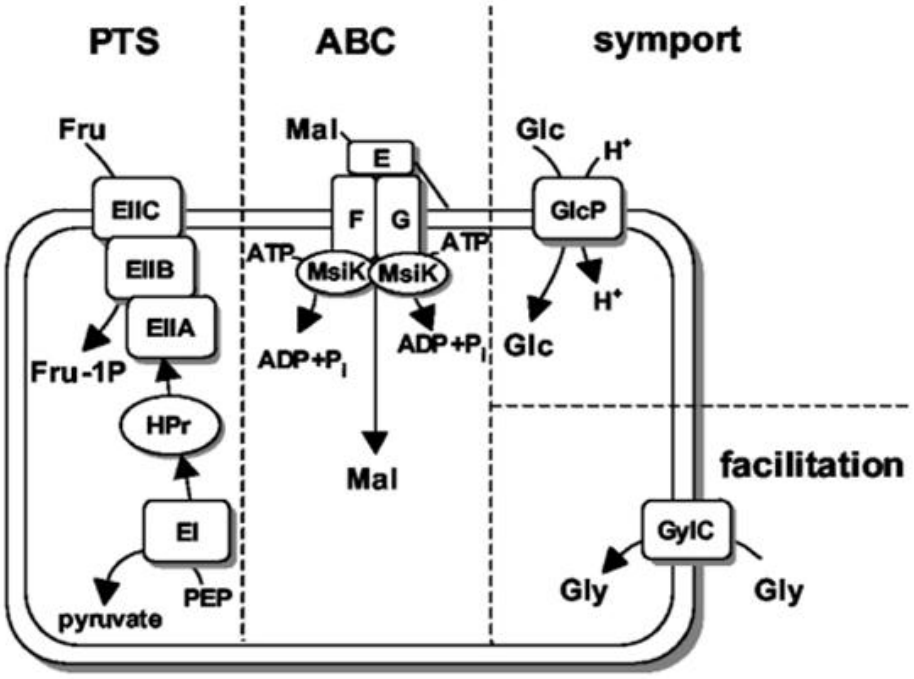


Figure 5.14 Carbon uptake systems known in *Streptomyces coelicolor*. Adopted from (Bertram *et al.*, 2004).

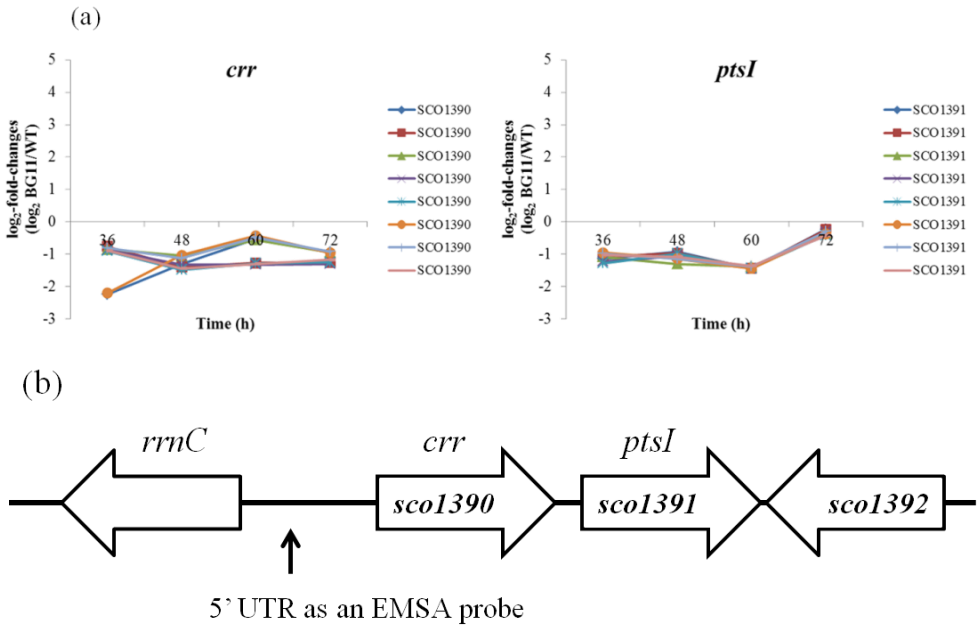


Figure 5.15 Expression patterns of IIA^{Crr} and EI. Eight probes per one gene showed similar expression patterns (a). All probes were down-regulated in BG11 compared to wild types. X-axis: time points, Y-axis: log_2-fold changes. Locations of genes and EMSA probe (b).

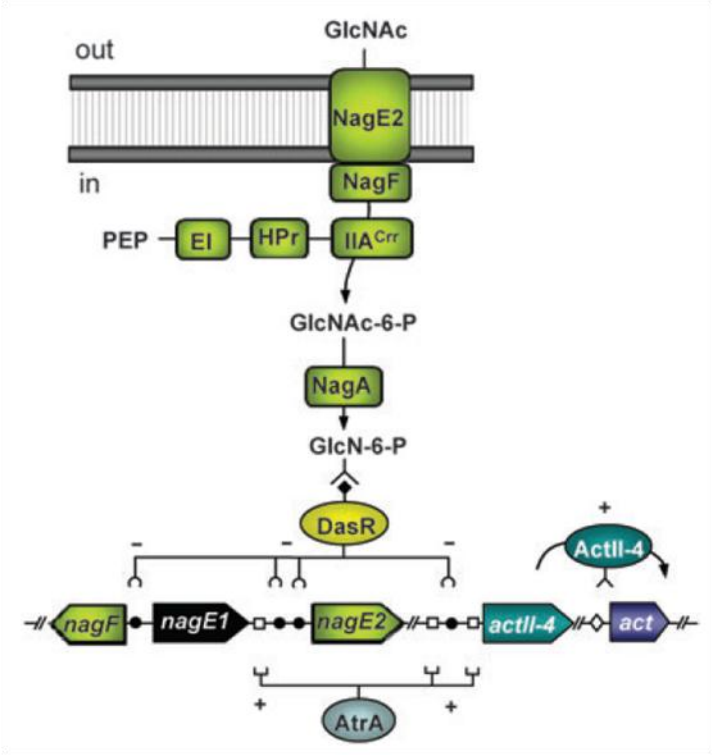


Figure 5.16 Model of GlcNAc-dependent signaling and uptake pathway. Glucosamine-6-phosphate is a key signal molecule in GlcNAc uptake system. Adopted from (Nothaft et al., 2010).

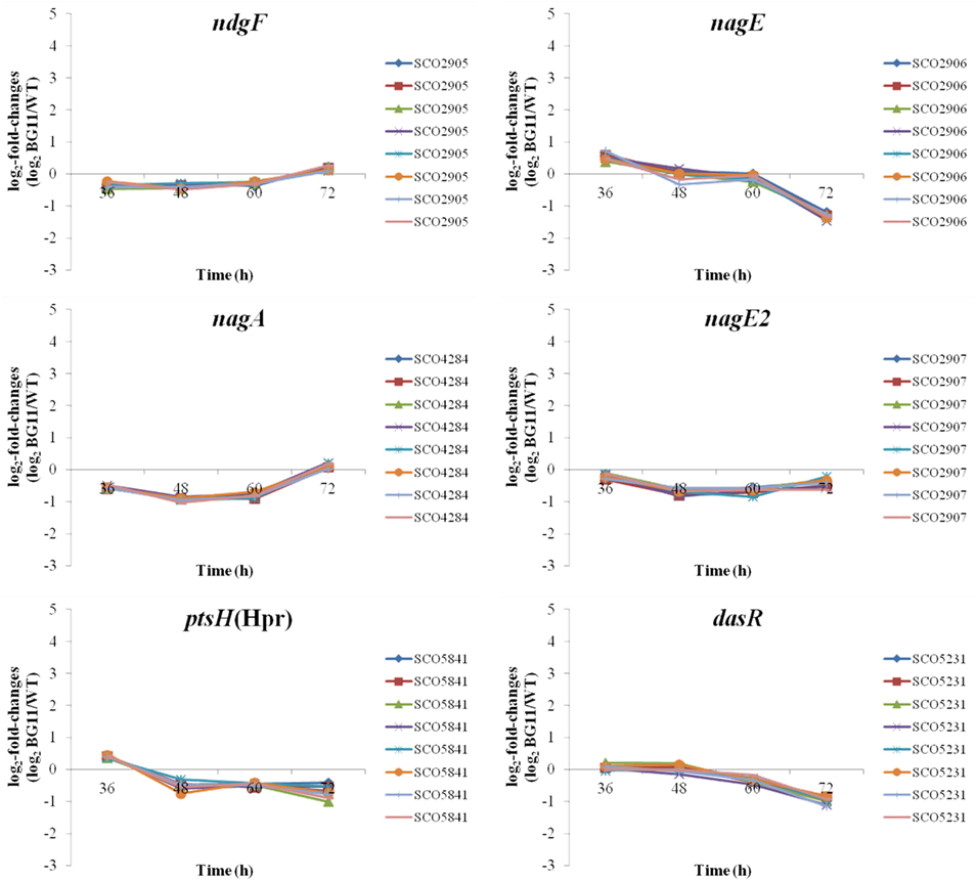


Figure 5.17 Expression pattern of genes related with PTS. Eight probes per one gene showed same expression patterns. All probes were down-regulated in BG11 compared to wild types. X-axis: time points, Y-axis: log_2-fold changes.

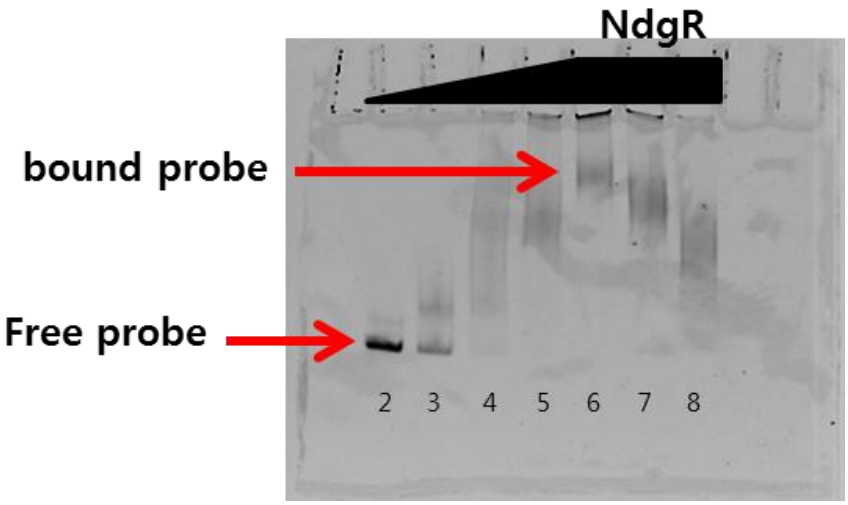


Figure 5.18 EMSA using the promoter sequence of IIA^{Crr} and EI. Lane 2, only probes (50 ng/μL) ; lane 3, probes and NdgR (1 μg); lane 4, probes and NdgR (2 μg); lane 5, probes and NdgR (4 μg); lane 6, probes and NdgR (10 μg); lane 7, probes, 50 ng of non-labeled probes as competitors, and NdgR (10 μg); lane 8, probes, 200 ng of non-labeled probes as competitors, and NdgR (10 μg).

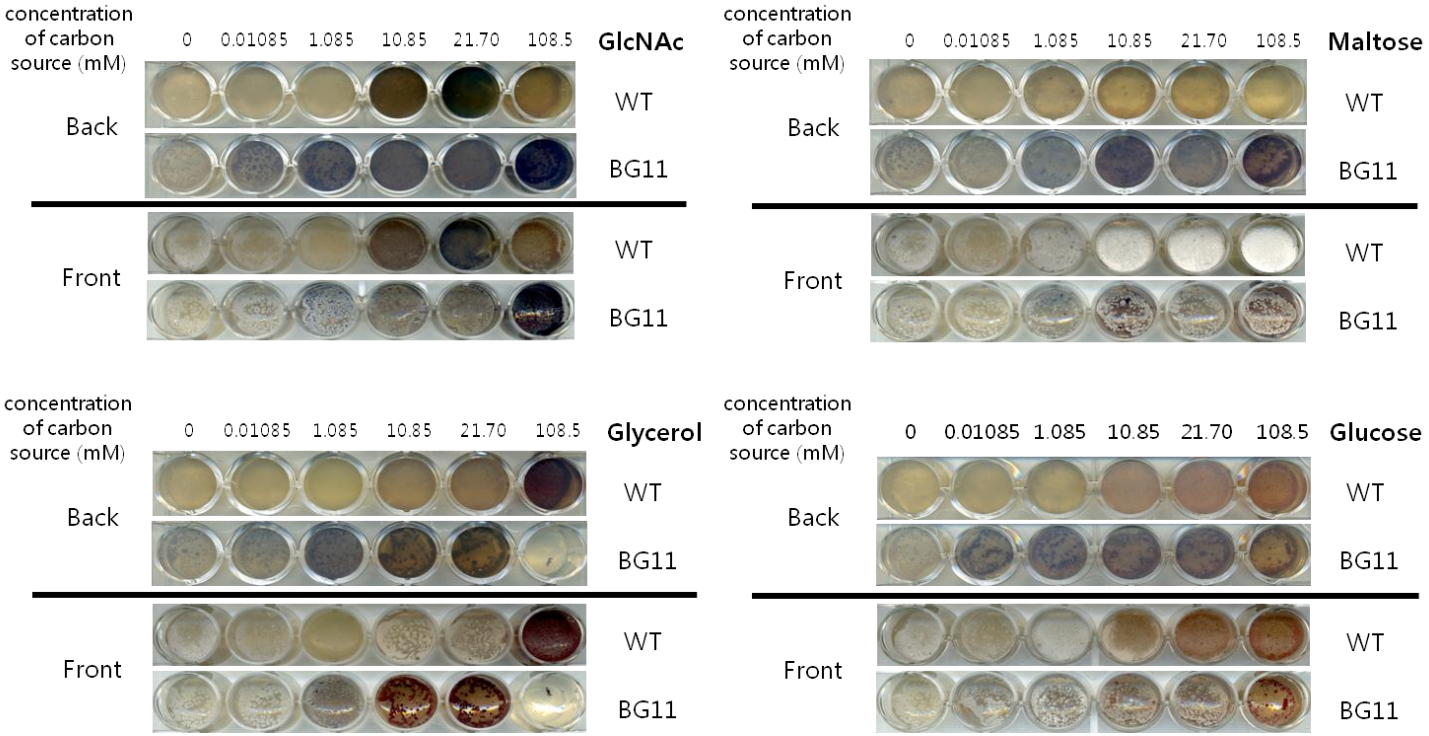


Figure 5.19 24-well plate cultures of wild type and BG11 grown in solid minimal media with ASN as a nitrogen-source and various carbon-sources in day 11. Various concentrations of carbon-source were used.

The other known carbohydrate uptake system also affected the growth and the antibiotics production in BG11. Except GlcNAc uptake system, genes related with glucose, maltose and glycerol uptake systems were up-regulated in BG11 (data not shown). Interestingly, BG11 produced antibiotics well in all condition (Figure 5.19). And in GlcNAc condition, the concentration of GlcNAc was not influenced by BG11, in contrast to wild type. Still, we do not know the exact mechanism to regulate carbohydrate uptake and utilization the system in BG11. However, based on the several clues such as target genes and physiological differences, it is clear that NdgR is involved in the carbon catabolism as well as the nitrogen metabolism.

According to the gene ontology data shown in Figure 5.6, most genes were related with the cell homeostasis. Homeostasis means the tendency to stay the equilibrium inside the cell. Cells strictly maintain their intracellular environment so that conditions remain optimal for the manufacturing and processing tasks that take place within against the stress from the outside.

Therefore, in the condition that was down-regulated genes related with cell homeostasis, BG11 should be severely weak against the outside stress. In addition, compared to the growth in the solid MM GlcNAc/ASN, BG11 rarely grew in the liquid MM GlcNAc/ASN (Figure 5.20). Normally we used shaking and glass beads to improve cell growth and antibiotic productions. However, the growth pattern of BG11 was quite different from the wild type strain and it showed aeration and physical stress is not helpful at all.

We tested cell viability using fluorescent dye staining. To analyze

viability, the ratio of live/dead cells was determined by staining nucleic acids with a combination of the green-fluorescent SYTO® 9 and the red-fluorescent PI (propidium iodide). The SYTO® 9 stains all kinds of cells but PI stains only cells that have damaged cell membranes or the dead cells. In Figure 5.21, the ratio of live/dead cells is shown. As expected, physical stress such as shaking with glass beads affected cell viability. In BG11, the proportion of live cells was larger in the condition without glass beads than in the condition of shaking with glass beads. We assumed that the percentage of live cells increased in the condition without physical stress because of slow growth and small ratio of dying cells. Fluorescent images observed by confocal microscope were also showed large ratio of live cells (shown in Figure 5.22). Then, we observed cell membranes directly by TEM to determine whether BG11 had really weak cell membranes compared with wild type or not. In complex $R5^-$ liquid media, wild type and BG11 did not show a big differences and this was the same observation of cell growth and the antibiotics production (Figure 5.23). However, in liquid MM GlcNAc/ASN, BG11 cell membrane was clearly defected. It was difficult to find the double-layered lipid membranes in BG11 and the amount and the size of cells were also smaller than wild type (Figure 5.24).

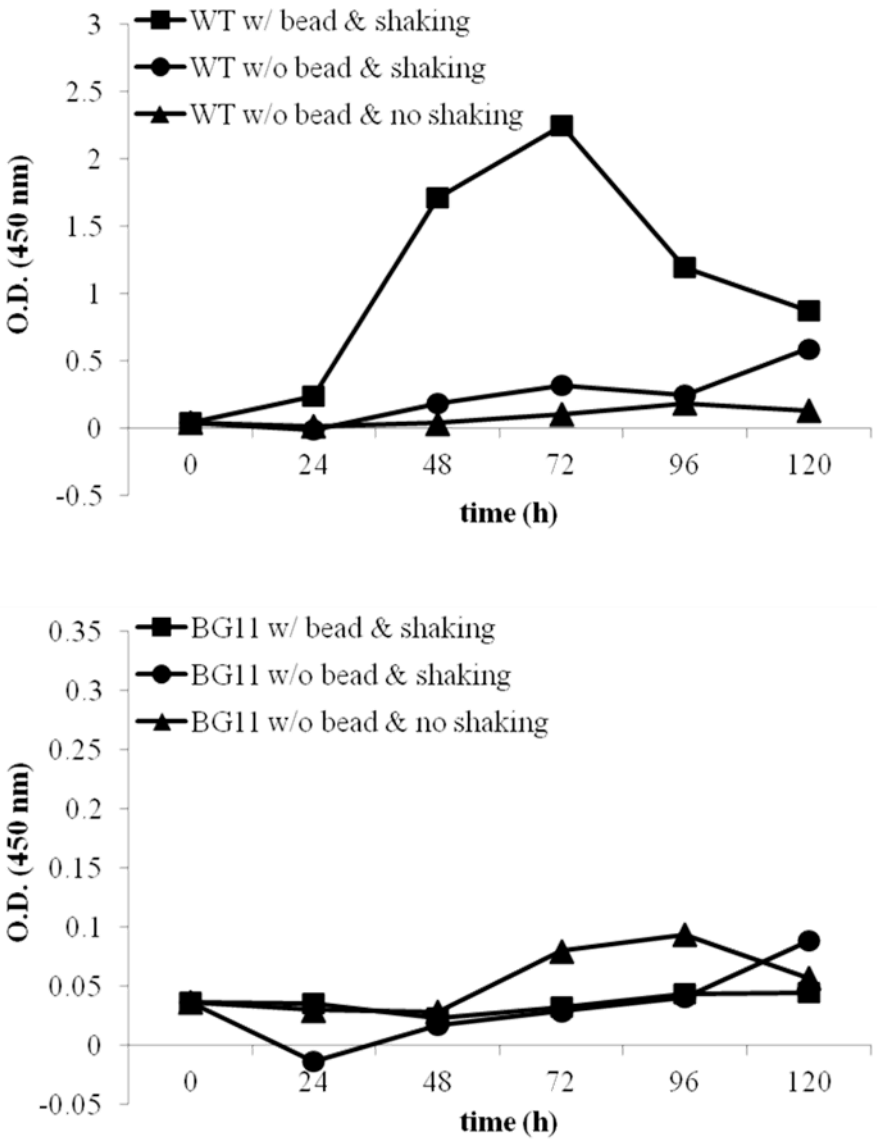
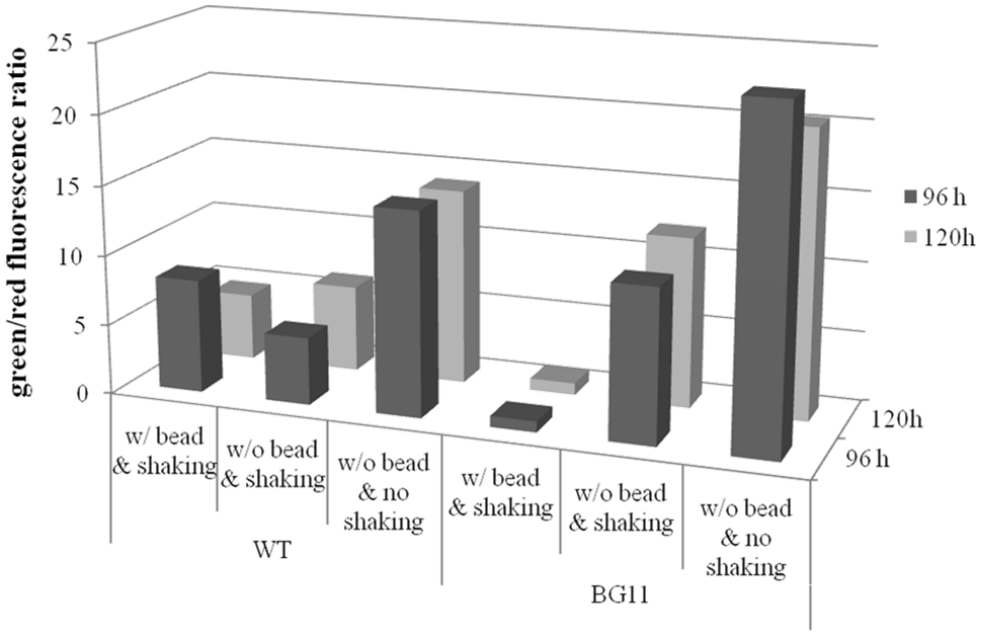


Figure 5.20 Growth curves of wild types and BG11 which were cultured in liquid MM GlcNAc/ASN. Average O.D. is observed at 450 nm.



		96 h	120h
Wild type	with beads & shaking	8.11	4.78
	without beads & shaking	4.82	6.20
	without beads & no shaking	14.60	14.00
BG11	with beads & shaking	0.79	0.85
	without beads & shaking	10.95	12.13
	without beads & no shaking	23.86	20.42

Figure 5.21 The fluorescence ratio of live/dead cells. Compared to wild types, BG11 was drastically decreased the ratio of live cells in the condition of the culture with beads and shaking.

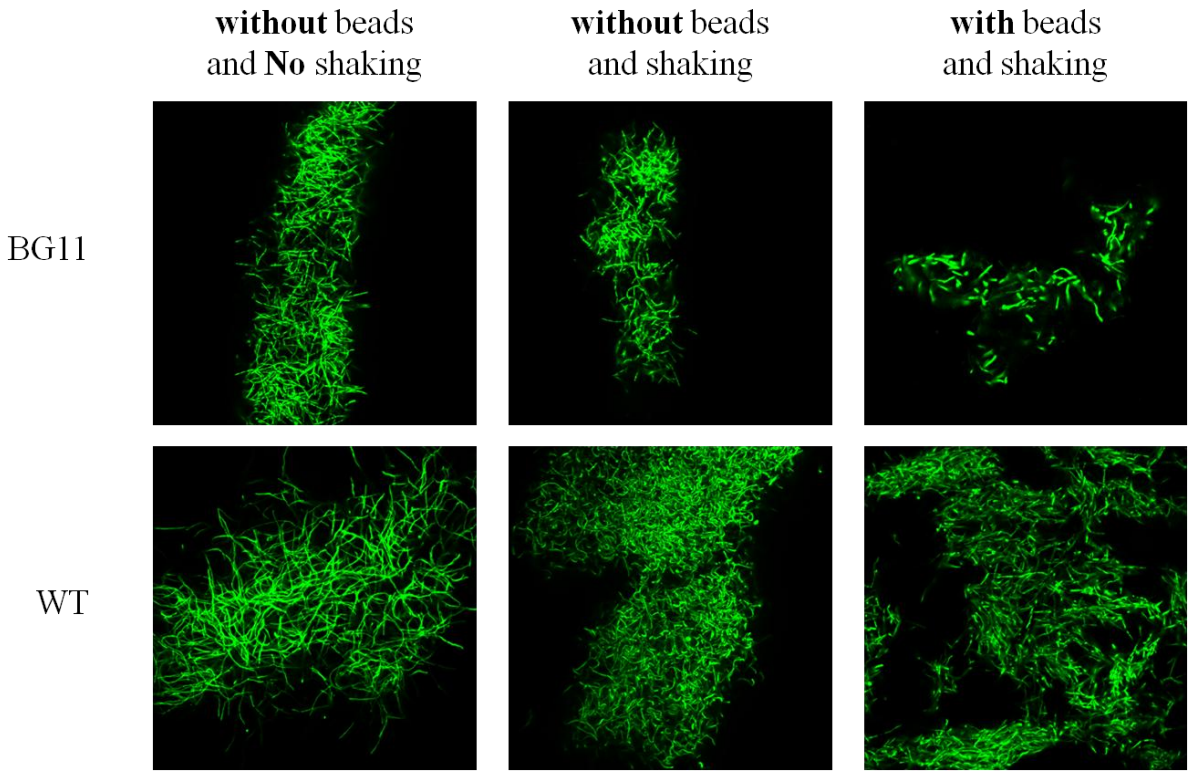


Figure 5.22 Fluorescence images of live cells at 96 h. BG11 cultured with beads and shaking was shown few live cells compared with other conditions.

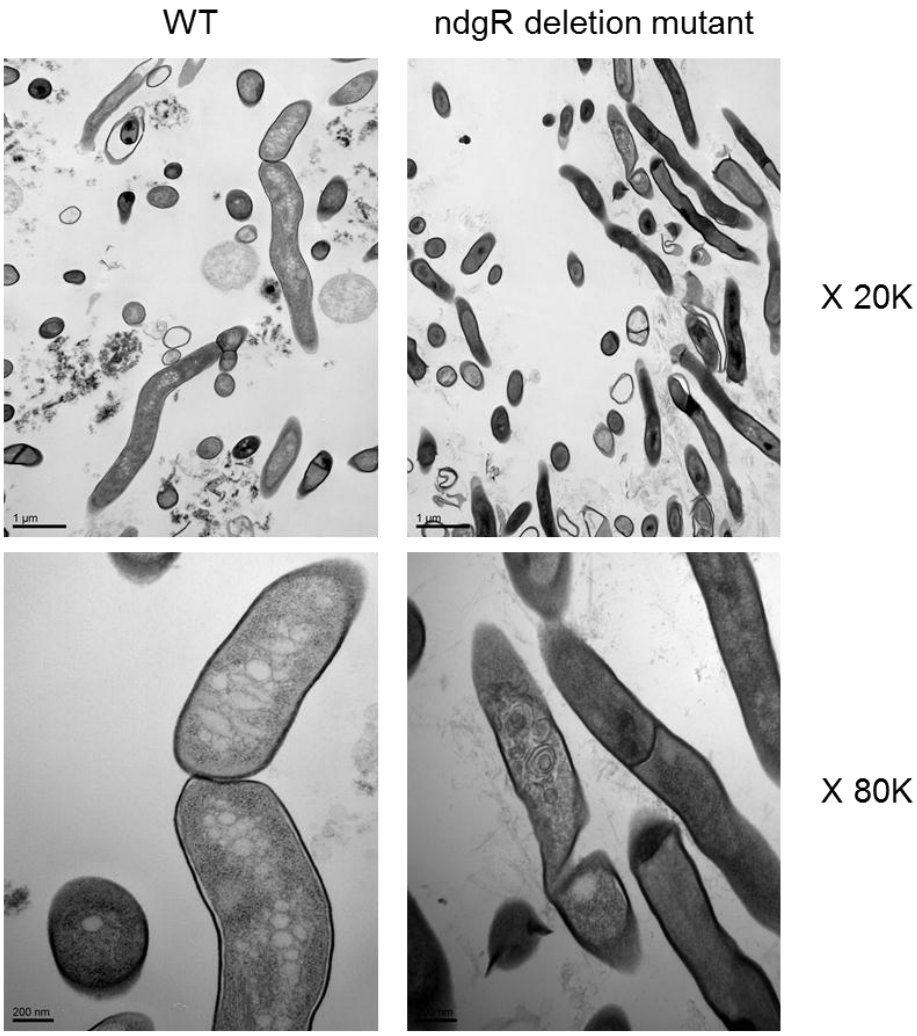


Figure 5.23 TEM images of wild type and BG11 grown in liquid $R5^-$ complex media. There were no big differences between wild type and BG11.

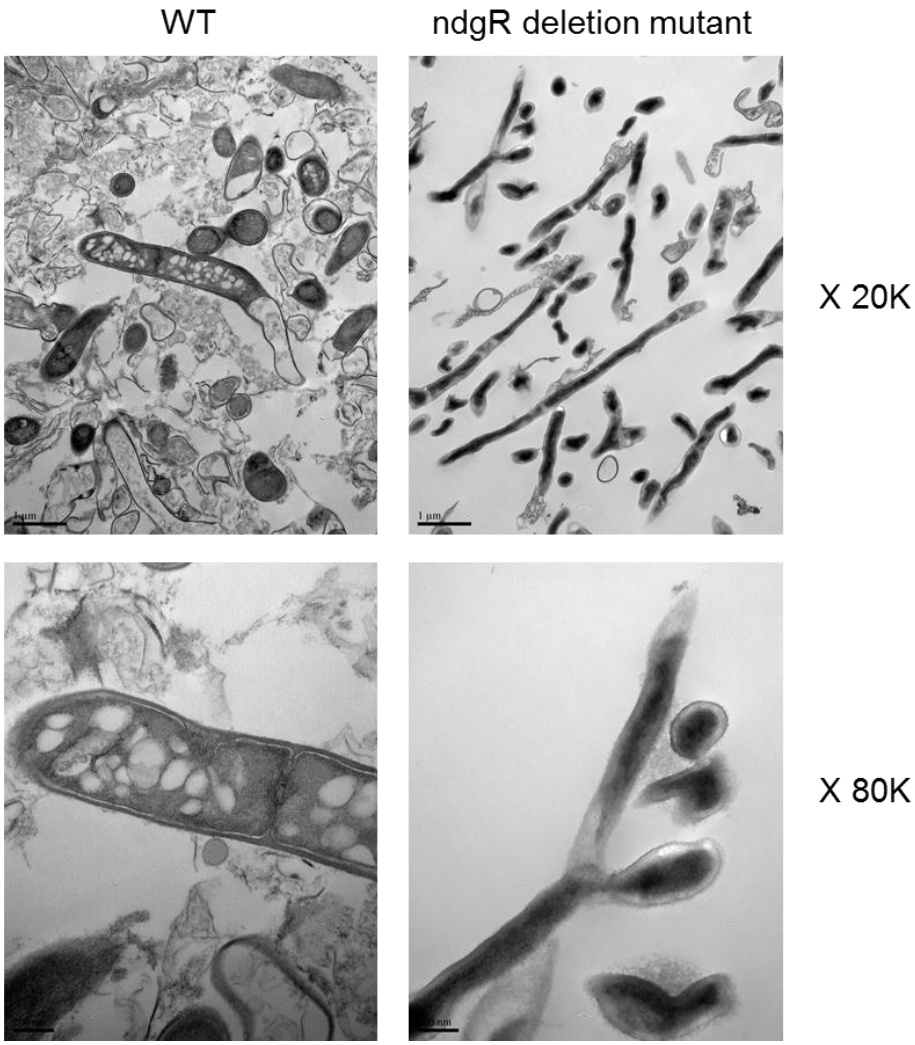


Figure 5.24 TEM images of wild type and BG11 grown in liquid MM GlcNAc/ASN. The defects of lipid membranes were observed in BG11.

5.3 Conclusion

NdgR was reported as a global regulator that controls amino acids synthesis, quorum sensing and antibiotics production in previous reports. In addition to those roles of NdgR, we newly revealed that NdgR also controls amino acids transport system, for example up-regulation of glutamate and branched amino acid transport system and down-regulation of methionine transport system, carbohydrate uptakes, and carbohydrate metabolism such as down-regulation of PTS, up-regulation of glycerol metabolism, cell stress response, and tolerance to toxic materials.

With the time course microarray experiment, we could reveal the new cellular functions of NdgR. In Figure 5.25, it is described the global roles of NdgR.

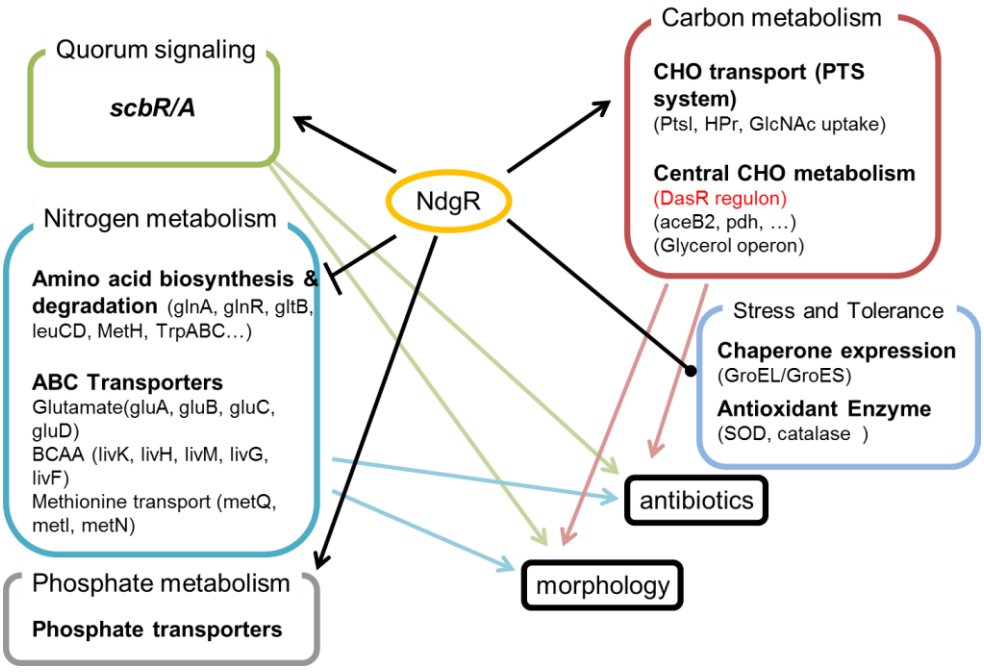


Figure 5.25 Hypothesis of NdgR targeted network.

Chapter VI

Conclusions and Recommendation

As the information of regulators gets larger, a regulatory network model would be constructed roughly at the present stage. However there are too many regulators in *Streptomyces* and complex cellular metabolism related with morphological development and secondary metabolism, it needs precise estimation and prediction to draw whole regulatory networks. In addition, antibiotics production is our main interest and this is quite unique feature in *Streptomyces*, therefore it is not easy to obtain the related information to other known bacterial regulatory network.

To overcome this problem, use of transcriptomic approach was successfully validated in analyzing transcriptional regulatory network for revealing hidden layer of regulatory network in this study. We used the microarray data of AbsB encodes an RNase III homolog and we showed known global regulator, AbsB controlled the lower level transcriptional regulator. Since AbsB which has a double-strand specific ribonucleolytic cleavage activity, targeted transcriptional regulators were regulated their mRNA expressions by ribonucleolytic cleavage. Comprehensive explanations on the changes of mRNA expression according to the presence of global regulator were suggested by transcriptomics approach and it successfully provided a clue for expanding transcriptional regulatory network.

Streptomyces is a natural antibiotics-producing genus of Actinobacteria. And overproduction of antibiotics of natural origin is one of the major issues to study *Streptomyces*. Therefore, we applied the transcriptional regulatory network analysis to over-produce antibiotics but to understand transcriptional regulatory network. From the microarray data of *afsS* disruption mutant, we found several transcriptional regulators which

showed transcriptionally independent expression patterns. Among them, SCO4677 was reported as a repressor of antibiotics production, and PhoU was newly identified as a repressor of antibiotics production. AfsS has already known as an activator of antibiotics production. The combination of the amplification of *afsS* and deletion of *sco4677* and amplification of *afsS* and deletion of *phoU* could show dramatic enhancement of antibiotics production. Therefore, we suggested an effective approach to increase antibiotics production and this approach would be possible to other targets of using transcriptional regulatory network.

In our group, many tools such as microarray, 2D-gel analysis, liquid chromatography, mass spectrometry combined with EMSA, SEM, TEM, RT-PCR and 96-well based minimal media culture assay (APM) with deletion mutants were applied for screening and identifying transcriptional regulators and their transcriptional networks. For further study in detail, time-course microarray experiments were performed using *ndgR* deletion mutants. Time series data of the mutant made us possible to estimate the cellular functions of transcriptional regulator more accurate. By time course data, we could perform clustering analysis and gene ontology analysis, then it could be identified that NdgR governed amino acids transport system, carbohydrate uptakes and carbohydrate metabolism, and cell stress response and tolerance to toxic materials addition to amino acids synthesis, quorum sensing and antibiotics production in previous report. Therefore, we could achieve a deeper level of comprehension that NdgR is an essential global regulator.

Still, the identification of transcriptional regulator and their regulatory network is ongoing. Recently, next generation sequencing techniques such

as RNA-seq, methylation-seq and ChIP-seq have newly developed. Therefore, it would be possible to pile up more precise and massive information about transcriptional regulators. Especially, we would be obtained the specific information about transcriptional regulator *in vivo* by ChIP-seq. Therefore, we need to become proficient these experiments to be applied in the major transcriptional regulators in the future. Moreover, the analysis method for internal metabolites in primary and secondary metabolism will give us clues on the change of metabolic flux caused by a regulator. More data of DNA chip datasets, RNA-seq and ChIP-seq produced by other groups are needed to integrate to our regulatory network and it will be possible to lead us the way of understanding *Streptomyces*.

REFERENCES

Aceti, D. J. & W. C. Champness, (1998) Transcriptional regulation of *Streptomyces coelicolor* pathway-specific antibiotic regulators by the *absA* and *absB* loci. *J Bacteriol* **180**: 3100-3106.

Adamidis, T. & W. Champness, (1992) Genetic analysis of *absB*, a *Streptomyces coelicolor* locus involved in global antibiotic regulation. *J Bacteriol* **174**: 4622-4628.

Amarasinghe, A. K., I. Calin-Jageman, A. Harmouch, W. Sun & A. W. Nicholson, (2001) *Escherichia coli* ribonuclease III: affinity purification of hexahistidine-tagged enzyme and assays for substrate binding and cleavage. *Methods Enzymol* **342**: 143-158.

Anderson, T. B., P. Brian & W. C. Champness, (2001) Genetic and transcriptional analysis of *absA*, an antibiotic gene cluster-linked two-component system that regulates multiple antibiotics in *Streptomyces coelicolor*. *Mol Microbiol* **39**: 553-566.

Barrett, T., D. B. Troup, S. E. Wilhite, P. Ledoux, D. Rudnev, C. Evangelista, I. F. Kim, A. Soboleva, M. Tomashevsky & R. Edgar, (2007) NCBI GEO: mining tens of millions of expression profiles--database and tools update. *Nucleic Acids Res* **35**: D760-765.

Baumbach, J., (2007) CoryneRegNet 4.0 - A reference database for corynebacterial gene regulatory networks. *BMC Bioinformatics* **8**: 429.

Bertram, R., M. Schlicht, K. Mahr, H. Nothaft, M. H. Saier, Jr. & F. Titgemeyer, (2004) In silico and transcriptional analysis of carbohydrate uptake systems of *Streptomyces coelicolor* A3(2). *J Bacteriol* **186**: 1362-1373.

Bibb, M. J., A. Domonkos, G. Chandra & M. J. Buttner, (2012) Expression of the chaplin and rodlin hydrophobic sheath proteins in *Streptomyces venezuelae* is controlled by sigma(BldN) and a cognate anti-sigma factor, RsbN. *Mol Microbiol* **84**: 1033-1049.

Birko, Z., S. Bialek, K. Buzas, E. Szajli, B. A. Traag, K. F. Medzihradszky, S. Rigali, E. Vijgenboom, A. Penyige, Z. Kele, G. P. van Wezel & S. Biro, (2007) The secreted signaling protein factor C triggers the A-factor response regulon in *Streptomyces griseus*: overlapping signaling routes. *Mol Cell Proteomics* **6**: 1248-1256.

Birko, Z., A. Sumegi, A. Vinnai, G. van Wezel, F. Szeszak, S. Vitalis, P. T. Szabo, Z. Kele, T. Janaky & S. Biro, (1999) Characterization of the gene for factor C, an extracellular signal protein involved in morphological differentiation of *Streptomyces griseus*. *Microbiology* **145 (9)**: 2245-2253.

Biro, S., Z. Birko & G. P. van Wezel, (2000) Transcriptional and functional analysis of the gene for factor C, an extracellular signal protein

involved in cytodifferentiation of *Streptomyces griseus*. *Antonie Van Leeuwenhoek* **78**: 277-285.

Blount, B. A., T. Weenink, S. Vasylechko & T. Ellis, (2012) Rational diversification of a promoter providing fine-tuned expression and orthogonal regulation for synthetic biology. *PLoS One* **7**: e33279.

Bolstad, B. M., R. A. Irizarry, M. Astrand & T. P. Speed, (2003) A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics* **19**: 185-193.

Brian, P., P. J. Riggle, R. A. Santos & W. C. Champness, (1996) Global negative regulation of *Streptomyces coelicolor* antibiotic synthesis mediated by an *absA*-encoded putative signal transduction system. *J Bacteriol* **178**: 3221-3231.

Bruheim, P., H. Sletta, M. J. Bibb, J. White & D. W. Levine, (2002) High-yield actinorhodin production in fed-batch culture by a *Streptomyces lividans* strain overexpressing the pathway-specific activator gene *actII-ORF4*. *J Ind Microbiol Biotechnol* **28**: 103-111.

Chakraburtty, R. & M. Bibb, (1997) The ppGpp synthetase gene (*relA*) of *Streptomyces coelicolor* A3(2) plays a conditional role in antibiotic production and morphological differentiation. *J Bacteriol* **179**: 5854-5861.

Chakraburtty, R., J. White, E. Takano & M. Bibb, (1996) Cloning, characterization and disruption of a (p)ppGpp synthetase gene (*relA*) of *Streptomyces coelicolor* A3(2). *Mol Microbiol* **19**: 357-368.

Chang, H.-M. C., Min-Yih; Shieh, Yi-Tien; Bibb, Mervyn J.; Chen, Carton W., (1996) The *cutRS* signal transduction system of *Streptomyces lividans* represses the biosynthesis of the polyketide antibiotic actinorhodin. *Mol Microbiol* **21**: 1075-1085.

Chater, K. F., (2001) Regulation of sporulation in *Streptomyces coelicolor* A3(2): a checkpoint multiplex? *Curr Opin Microbiol* **4**: 667-673.

Chater, K. F., (2006) Streptomyces inside-out: a new perspective on the bacteria that provide us with antibiotics. *Philos Trans R Soc Lond B Biol Sci* **361**: 761-768.

Chen, L., Y. Lu, J. Chen, W. Zhang, D. Shu, Z. Qin, S. Yang & W. Jiang, (2008) Characterization of a negative regulator AveI for avermectin biosynthesis in *Streptomyces avermitilis* NRRL8165. *Appl Microbiol Biotechnol* **80**: 277-286.

Cho, B. K., K. Zengler, Y. Qiu, Y. S. Park, E. M. Knight, C. L. Barrett, Y. Gao & B. O. Palsson, (2009) The transcription unit architecture of the *Escherichia coli* genome. *Nat Biotechnol* **27**: 1043-1049.

Corre, C., L. Song, S. O'Rourke, K. F. Chater & G. L. Challis, (2008) 2-

Alkyl-4-hydroxymethylfuran-3-carboxylic acids, antibiotic production inducers discovered by *Streptomyces coelicolor* genome mining. *Proc Natl Acad Sci U S A* **105**: 17510-17515.

Dairi, T., K. Aisaka, R. Katsumata & M. Hasegawa, (1995) A self-defense gene homologous to tetracycline effluxing gene essential for antibiotic production in *Streptomyces aureofaciens*. *Biosci Biotechnol Biochem* **59**: 1835-1841.

den Hengst, C. D., N. T. Tran, M. J. Bibb, G. Chandra, B. K. Leskiw & M. J. Buttner, (2010) Genes essential for morphological development and antibiotic production in *Streptomyces coelicolor* are targets of BldD during vegetative growth. *Mol Microbiol* **78**: 361-379.

Edgar, R., M. Domrachev & A. E. Lash, (2002) Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res* **30**: 207-210.

Feitelson, J. S., F. Malpartida & D. A. Hopwood, (1985) Genetic and biochemical characterization of the red gene cluster of *Streptomyces coelicolor* A3(2). *J Gen Microbiol* **131**: 2431-2441.

Fernandez-Moreno, M. A., J. L. Caballero, D. A. Hopwood & F. Malpartida, (1991) The *act* cluster contains regulatory and antibiotic export genes, direct targets for translational control by the *bldA* tRNA gene of *Streptomyces*. *Cell* **66**: 769-780.

Fiehn, O., (2001) Combining genomics, metabolome analysis, and biochemical modelling to understand metabolic networks. *Comp Funct Genomics* **2**: 155-168.

Flardh, K. & M. J. Buttner, (2009) *Streptomyces* morphogenetics: dissecting differentiation in a filamentous bacterium. *Nat Rev Microbiol* **7**: 36-49.

Floriano, B. & M. Bibb, (1996) *afsR* is a pleiotropic but conditionally required regulatory gene for antibiotic production in *Streptomyces coelicolor* A3(2). *Mol Microbiol* **21**: 385-396.

Gramajo, H. C., E. Takano & M. J. Bibb, (1993) Stationary-phase production of the antibiotic actinorhodin in *Streptomyces coelicolor* A3(2) is transcriptionally regulated. *Mol Microbiol* **7**: 837-845.

Guilfoile, P. G. & C. R. Hutchinson, (1991) A bacterial analog of the mdr gene of mammalian tumor cells is present in *Streptomyces peucetius*, the producer of daunorubicin and doxorubicin. *Proc Natl Acad Sci U S A* **88**: 8553-8557.

Guo, T., J. Kong, L. Zhang, C. Zhang & S. Hu, (2012) Fine Tuning of the Lactate and Diacetyl Production through Promoter Engineering in *Lactococcus lactis*. *PLoS One* **7**: e36296.

Gust, B., G. L. Challis, K. Fowler, T. Kieser & K. F. Chater, (2003) PCR-

targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. *Proc Natl Acad Sci U S A* **100**: 1541-1546.

Guthrie, E. P., C. S. Flaxman, J. White, D. A. Hodgson, M. J. Bibb & K. F. Chater, (1998) A response-regulator-like activator of antibiotic synthesis from *Streptomyces coelicolor* A3(2) with an amino-terminal domain that lacks a phosphorylation pocket. *Microbiology* **144 (3)**: 727-738.

Hesketh, A., H. Kock, S. Mootien & M. Bibb, (2009) The role of *absC*, a novel regulatory gene for secondary metabolism, in zinc-dependent antibiotic production in *Streptomyces coelicolor* A3(2). *Mol Microbiol* **74**: 1427-1444.

Higo, A., H. Hara, S. Horinouchi & Y. Ohnishi, (2012) Genome-wide Distribution of AdpA, a Global Regulator for Secondary Metabolism and Morphological Differentiation in *Streptomyces*, Revealed the Extent and Complexity of the AdpA Regulatory Network. *DNA research* **19**: 259-274.

Hindle, Z. & C. P. Smith, (1994) Substrate induction and catabolite repression of the *Streptomyces coelicolor* glycerol operon are mediated through the GylR protein. *Mol Microbiol* **12**: 737-745.

Hirano, S., K. Tanaka, Y. Ohnishi & S. Horinouchi, (2008) Conditionally positive effect of the TetR-family transcriptional regulator AtrA on streptomycin production by *Streptomyces griseus*. *Microbiology* **154**: 905-914.

Horinouchi, S., (2003) AfsR as an integrator of signals that are sensed by multiple serine/threonine kinases in *Streptomyces coelicolor* A3(2). *J Ind Microbiol Biotechnol* **30**: 462-467.

Horinouchi, S., M. Kito, M. Nishiyama, K. Furuya, S. K. Hong, K. Miyake & T. Beppu, (1990) Primary structure of AfsR, a global regulatory protein for secondary metabolite formation in *Streptomyces coelicolor* A3(2). *Gene* **95**: 49-56.

Hosaka, T., M. Ohnishi-Kameyama, H. Muramatsu, K. Murakami, Y. Tsurumi, S. Kodani, M. Yoshida, A. Fujie & K. Ochi, (2009) Antibacterial discovery in actinomycetes strains with mutations in RNA polymerase or ribosomal protein S12. *Nat Biotechnol* **27**: 462-464.

Huang da, W., B. T. Sherman & R. A. Lempicki, (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat protoc* **4**: 44-57.

Huang, J., J. Shi, V. Molle, B. Sohlberg, D. Weaver, M. J. Bibb, N. Karoonuthaisiri, C. J. Lih, C. M. Kao, M. J. Buttner & S. N. Cohen,

(2005) Cross-regulation among disparate antibiotic biosynthetic pathways of *Streptomyces coelicolor*. *Mol Microbiol* **58**: 1276-1287.
Hwang, D., A. G. Rust, S. Ramsey, J. J. Smith, D. M. Leslie, A. D. Weston, P. de Atauri, J. D. Aitchison, L. Hood, A. F. Siegel & H. Bolouri, (2005a) A data integration methodology for systems biology. *Proc Natl Acad Sci U S A* **102**: 17296-17301.
Hwang, D., J. J. Smith, D. M. Leslie, A. D. Weston, A. G. Rust, S. Ramsey, P. de Atauri, A. F. Siegel, H. Bolouri, J. D. Aitchison & L. Hood, (2005b) A data integration methodology for systems biology: experimental verification. *Proc Natl Acad Sci U S A* **102**: 17302-17307.
Ishizuka, H., S. Horinouchi, H. M. Kieser, D. A. Hopwood & T. Beppu, (1992) A putative two-component regulatory system involved in secondary metabolism in *Streptomyces* spp. *J Bacteriol* **174**: 7585-7594.
Jensen, P. R. & K. Hammer, (1998) Artificial promoters for metabolic optimization. *Biotechnol Bioeng* **58**: 191-195.
Joo, H. S., Y. H. Yang, C. S. Lee, J. H. Kim & B. G. Kim, (2007) Fragmentation study on butanolides with tandem mass spectrometry and its application for the screening of ScbR-captured quorum sensing molecules in *Streptomyces coelicolor* A3(2). *Rapid Commun Mass Spectrom* **21**: 764-770.
Joyce, A. R. & B. O. Palsson, (2006) The model organism as a system: integrating 'omics' data sets. *Nat Rev Mol Cell Biol* **7**: 198-210.
Kandpal, R., B. Saviola & J. Felton, (2009) The era of 'omics unlimited. *Biotechniques* **46**: 351-352, 354-355.
Kang, S. H., J. Huang, H. N. Lee, Y. A. Hur, S. N. Cohen & E. S. Kim, (2007) Interspecies DNA microarray analysis identifies WblA as a pleiotropic down-regulator of antibiotic biosynthesis in *Streptomyces*. *J Bacteriol* **189**: 4315-4319.
Kato, J. Y., N. Funa, H. Watanabe, Y. Ohnishi & S. Horinouchi, (2007) Biosynthesis of gamma-butyrolactone autoregulators that switch on secondary metabolism and morphological development in *Streptomyces*. *Proc Natl Acad Sci U S A* **104**: 2378-2383.
Kelemen, G. H. & M. J. Buttner, (1998) Initiation of aerial mycelium formation in *Streptomyces*. *Curr Opin Microbiol* **1**: 656-662.
Kelly, K. S., K. Ochi & G. H. Jones, (1991) Pleiotropic effects of a *relC* mutation in *Streptomyces antibioticus*. *J Bacteriol* **173**: 2297-2300.
Kieser, T., M. J. Bibb, M. J. Buttner, K. Chater & D. A. Hopwood, (2000) *Practical Streptomyces Genetics*. John Innes Centre, Norwich Research Park, Colney, Norwich, UK.
Kim, E. S., J. Y. Song, D. W. Kim, K. F. Chater & K. J. Lee, (2008) A possible extended family of regulators of sigma factor activity in

Streptomyces coelicolor. *J Bacteriol* **190**: 7559-7566.
Kurumbang, N. P., K. Liou & J. K. Sohng, (2010) Biosynthesis of paromamine derivatives in engineered *Escherichia coli* by heterologous expression. *J Appl Microbiol* **108**: 1780-1788.
Lai, C., J. Xu, Y. Tozawa, Y. Okamoto-Hosoya, X. Yao & K. Ochi, (2002) Genetic and physiological characterization of *rpoB* mutations that activate antibiotic production in *Streptomyces lividans*. *Microbiology* **148**: 3365-3373.
Lee, H. N., J. Huang, J. H. Im, S. H. Kim, J. H. Noh, S. N. Cohen & E. S. Kim, (2010) Putative TetR family transcriptional regulator SCO1712 encodes an antibiotic downregulator in *Streptomyces coelicolor*. *Appl Environ Microbiol* **76**: 3039-3043.
Lee, P.-C., T. Umeyama & S. Horinouchi, (2002) *afsS* is a target of AfsR, a transcriptional factor with ATPase activity that globally controls secondary metabolism in *Streptomyces coelicolor* A3(2). *Mol Microbiol* **43**: 1413-1430.
Lewis, R. A., S. K. Shahi, E. Laing, G. Bucca, G. Efthimiou, M. Bushell & C. P. Smith, (2011) Genome-wide transcriptomic analysis of the response to nitrogen limitation in *Streptomyces coelicolor* A3(2). *BMC Res Notes* **4**: 78.
Li, R. & C. A. Townsend, (2006) Rational strain improvement for enhanced clavulanic acid production by genetic engineering of the glycolytic pathway in *Streptomyces clavuligerus*. *Metab Eng* **8**: 240-252.
Li, W. Y., Xin; Guo, Yuzheng; Yu, Zhen; Zhou, Xiufen; Deng, Zixin; Kieser, Helen; Chater, Keith F.; Tao, Meifeng, (2006) Identification of a gene negatively affecting antibiotic production and morphological differentiation in *Streptomyces coelicolor* A3(2). *J Bacteriol* **188**: 8368-8375.
Lian, W., K. P. Jayapal, S. Charaniya, S. Mehra, F. Glod, Y. S. Kyung, D. H. Sherman & W. S. Hu, (2008) Genome-wide transcriptome analysis reveals that a pleiotropic antibiotic regulator, AfsS, modulates nutritional stress response in *Streptomyces coelicolor* A3(2). *BMC Genomics* **9**: 56.
Lin, J. & J. Qian, (2007) Systems biology approach to integrative comparative genomics. *Expert Rev Proteomics* **4**: 107-119.
Lockhart, D. J., H. Dong, M. C. Byrne, M. T. Follettie, M. V. Gallo, M. S. Chee, M. Mittmann, C. Wang, M. Kobayashi, H. Horton & E. L. Brown, (1996) Expression monitoring by hybridization to high-density oligonucleotide arrays. *Nat Biotechnol* **14**: 1675-1680.
Lu, Y. W., Weihua; Shu, Dan; Zhang, Weiwen; Chen, Lei; Qin, Zhongjun; Yang, Sheng; Jiang, Weihong, (2007) Characterization of a novel

two-component regulatory system involved in the regulation of both actinorhodin and a type I polyketide in *Streptomyces coelicolor*. *Appl. Microbiol. Biotechnol.* **77**: 625-635.

Malla, S., N. P. Niraula, K. Liou & J. K. Sohng, (2010) Self-resistance mechanism in *Streptomyces peucetius*: overexpression of *drrA*, *drrB* and *drrC* for doxorubicin enhancement. *Microbiol Res* **165**: 259-267.

Mardis, E. R., (2007) ChIP-seq: welcome to the new frontier. *Nat methods* **4**: 613-614.

Marioni, J. C., C. E. Mason, S. M. Mane, M. Stephens & Y. Gilad, (2008) RNA-seq: an assessment of technical reproducibility and comparison with gene expression arrays. *Genome Res* **18**: 1509-1517.

Martin, J. F. & P. Liras, (2010) Engineering of regulatory cascades and networks controlling antibiotic biosynthesis in *Streptomyces*. *Curr Opin Microbiol* **13**: 263-273.

Martin, J. F., A. Sola-Landa, F. Santos-Beneit, L. T. Fernandez-Martinez, C. Prieto & A. Rodriguez-Garcia, (2011) Cross-talk of global nutritional regulators in the control of primary and secondary metabolism in *Streptomyces*. *Microb Biotechnol* **4**: 165-174.

Matsumoto, A., S. K. Hong, H. Ishizuka, S. Horinouchi & T. Beppu, (1994) Phosphorylation of the AfsR protein involved in secondary metabolism in *Streptomyces* species by a eukaryotic-type protein kinase. *Gene* **146**: 47-56.

McKenzie, N. L. & J. R. Nodwell, (2007) Phosphorylated AbsA2 negatively regulates antibiotic production in *Streptomyces coelicolor* through interactions with pathway-specific regulatory gene promoters. *J Bacteriol* **189**: 5284-5292.

Mendes, M. V., S. Tunca, N. Anton, E. Recio, A. Sola-Landa, J. F. Aparicio & J. F. Martin, (2007) The two-component *phoR-phoP* system of *Streptomyces natalensis*: Inactivation or deletion of *phoP* reduces the negative phosphate regulation of pimaricin biosynthesis. *Metab Eng* **9**: 217-227.

Metzker, M. L., (2010) Sequencing technologies - the next generation. *Nat Rev Genet* **11**: 31-46.

Murakami, T., J. Burian, K. Yanai, M. J. Bibb & C. J. Thompson, (2011) A system for the targeted amplification of bacterial gene clusters multiplies antibiotic yield in *Streptomyces coelicolor*. *Proc Natl Acad Sci U S A* **108**: 16020-16025.

Narva, K. E. & J. S. Feitelson, (1990) Nucleotide sequence and transcriptional analysis of the *redD* locus of *Streptomyces*

coelicolor A3(2). *J Bacteriol* **172**: 326-333.
Nothaft, H., S. Rigali, B. Boomsma, M. Swiatek, K. J. McDowall, G. P. van Wezel & F. Titgemeyer, (2010) The permease gene *nagE2* is the key to N-acetylglucosamine sensing and utilization in *Streptomyces coelicolor* and is subject to multi-level control. *Mol Microbiol* **75**: 1133-1144.
O'Rourke, S., A. Wietzorrek, K. Fowler, C. Corre, G. L. Challis & K. F. Chater, (2009) Extracellular signalling, translational control, two repressors and an activator all contribute to the regulation of methylenomycin production in *Streptomyces coelicolor*. *Mol Microbiol* **71**: 763-778.
Ochi, K., (1990a) A relaxed (*rel*) mutant of *Streptomyces coelicolor* A3(2) with a missing ribosomal protein lacks the ability to accumulate ppGpp, A-factor and prodigiosin. *J Gen Microbiol* **136**: 2405-2412.
Ochi, K., (1990b) *Streptomyces relC* mutants with an altered ribosomal protein ST-L11 and genetic analysis of a *Streptomyces griseus relC* mutant. *J Bacteriol* **172**: 4008-4016.
Ohnishi Y Fau - Kameyama, S., H. Kameyama S Fau - Onaka, S. Onaka H Fau - Horinouchi & S. Horinouchi, (1999) The A-factor regulatory cascade leading to streptomycin biosynthesis in *Streptomyces griseus*: identification of a target gene of the A-factor receptor. *Mol, Microbiol.* **34 (1)**: 102-111
Olano, C., F. Lombo, C. Mendez & J. A. Salas, (2008) Improving production of bioactive secondary metabolites in actinomycetes by metabolic engineering. *Metab Eng* **10**: 281-292.
Onaka, H., N. Ando, T. Nihira, Y. Yamada, T. Beppu & S. Horinouchi, (1995) Cloning and characterization of the A-factor receptor gene from *Streptomyces griseus*. *J Bacteriol* **177**: 6083-6092.
Onaka, H. & S. Horinouchi, (1997) DNA-binding activity of the A-factor receptor protein and its recognition DNA sequences. *Mol Microbiol* **24**: 991-1000.
Onaka, H., T. Nakagawa & S. Horinouchi, (1998) Involvement of two A-factor receptor homologues in *Streptomyces coelicolor* A3(2) in the regulation of secondary metabolism and morphogenesis. *Mol Microbiol* **28**: 743-753.
Ou, X. Z., Bo; Zhang, Lin; Zhao, Guoping; Ding, Xiaoming, (2009) Characterization of *rrdA*, a TetR family protein gene involved in the regulation of secondary metabolism in *Streptomyces coelicolor*. *Appl. Environ. Microbiol.* **75**: 2158-2165.
Paget, M. S., J. G. Kang, J. H. Roe & M. J. Buttner, (1998) sigmaR, an RNA polymerase sigma factor that modulates expression of the

thioredoxin system in response to oxidative stress in *Streptomyces coelicolor* A3(2). *EMBO J* **17**: 5776-5782.

Park, J. W., S. R. Park, K. K. Nepal, A. R. Han, Y. H. Ban, Y. J. Yoo, E. J. Kim, E. M. Kim, D. Kim, J. K. Sohng & Y. J. Yoon, (2011) Discovery of parallel pathways of kanamycin biosynthesis allows antibiotic manipulation. *Nat Chem Biol* **7**: 843-852.

Park, S. S., Y. H. Yang, E. Song, E. J. Kim, W. S. Kim, J. K. Sohng, H. C. Lee, K. K. Liou & B. G. Kim, (2009) Mass spectrometric screening of transcriptional regulators involved in antibiotic biosynthesis in *Streptomyces coelicolor* A3(2). *J Ind Microbiol Biotechnol* **36**: 1073-1083.

Pawlik, K., M. Kotowska, K. F. Chater, K. Kuczek & E. Takano, (2007) A cryptic type I polyketide synthase (*cpk*) gene cluster in *Streptomyces coelicolor* A3(2). *Arch Microbiol* **187**: 87-99.

Perez-Llarena, F. J., P. Liras, A. Rodriguez-Garcia & J. F. Martin, (1997) A regulatory gene (*ccaR*) required for cephamycin and clavulanic acid production in *Streptomyces clavuligerus*: amplification results in overproduction of both beta-lactam compounds. *J Bacteriol* **179**: 2053-2059.

Potuckova, L., G. H. Kelemen, K. C. Findlay, M. A. Lonetto, M. J. Buttner & J. Kormanec, (1995) A new RNA polymerase sigma factor, sigma F, is required for the late stages of morphological differentiation in *Streptomyces* spp. *Mol Microbiol* **17**: 37-48.

Price, B., T. Adamidis, R. Kong & W. Champness, (1999) A *Streptomyces coelicolor* antibiotic regulatory gene, *absB*, encodes an RNase III homolog. *J Bacteriol* **181**: 6142-6151.

Pullan, S. T., G. Chandra, M. J. Bibb & M. Merrick, (2011) Genome-wide analysis of the role of GlnR in *Streptomyces venezuelae* provides new insights into global nitrogen regulation in actinomycetes. *BMC Genomics* **12**: 175.

Qiu, J., Y. Zhuo, D. Zhu, X. Zhou, L. Zhang, L. Bai & Z. Deng, (2011) Overexpression of the ABC transporter AvtAB increases avermectin production in *Streptomyces avermitilis*. *Appl Microbiol Biotechnol* **92**: 337-345.

Rigali, S., H. Nothaft, E. E. Noens, M. Schlicht, S. Colson, M. Muller, B. Joris, H. K. Koerten, D. A. Hopwood, F. Titgemeyer & G. P. van Wezel, (2006) The sugar phosphotransferase system of *Streptomyces coelicolor* is regulated by the GntR-family regulator DasR and links N-acetylglucosamine metabolism to the control of development. *Mol Microbiol* **61**: 1237-1251.

Rigali, S., F. Titgemeyer, S. Barends, S. Mulder, A. W. Thomae, D. A.

Hopwood & G. P. van Wezel, (2008) Feast or famine: the global regulator DasR links nutrient stress to antibiotic production by *Streptomyces*. *EMBO Rep* **9**: 670-675.

Ryding, N. J., T. B. Anderson & W. C. Champness, (2002) Regulation of the *Streptomyces coelicolor* calcium-dependent antibiotic by *absA*, encoding a cluster-linked two-component system. *J Bacteriol* **184**: 794-805.

Ryu, Y. G., E. S. Kim, D. W. Kim, S. K. Kim & K. J. Lee, (2007) Differential stringent responses of *Streptomyces coelicolor* M600 to starvation of specific nutrients. *J Microbiol Biotechnol* **17**: 305-312.

Santos-Beneit, F., A. Rodríguez-García, A. Sola-Landa & J. F. Martín, (2009) Cross-talk between two global regulators in *Streptomyces*: PhoP and AfsR interact in the control of *afsS*, *pstS* and *phoRP* transcription. *Mol Microbiol* **72**: 53-68.

Santos-Beneit, F., A. Rodriguez-Garcia & J. F. Martin, (2011) Complex transcriptional control of the antibiotic regulator *afsS* in *Streptomyces*: PhoP and AfsR are overlapping, competitive activators. *J Bacteriol* **193**: 2242-2251.

Santos-Beneit, F., A. Rodriguez-Garcia & J. F. Martin, (2012) Overlapping binding of PhoP and AfsR to the promoter region of *glnR* in *Streptomyces coelicolor*. *Microbiol Res*.

Sawai, R., A. Suzuki, Y. Takano, P. C. Lee & S. Horinouchi, (2004) Phosphorylation of AfsR by multiple serine/threonine kinases in *Streptomyces coelicolor* A3(2). *Gene* **334**: 53-61.

Schena, M., D. Shalon, R. W. Davis & P. O. Brown, (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* **270**: 467-470.

Sheeler, N. L., S. V. MacMillan & J. R. Nodwell, (2005) Biochemical activities of the *absA* two-component system of *Streptomyces coelicolor*. *J Bacteriol* **187**: 687-696.

Shu, D., L. Chen, W. Wang, Z. Yu, C. Ren, W. Zhang, S. Yang, Y. Lu & W. Jiang, (2009) *afsQ1-Q2-sigQ* is a pleiotropic but conditionally required signal transduction system for both secondary metabolism and morphological development in *Streptomyces coelicolor*. *Appl Microbiol Biotechnol* **81**: 1149-1160.

Singh, O. V. & N. S. Nagaraj, (2006) Transcriptomics, proteomics and interactomics: unique approaches to track the insights of bioremediation. *Brief Funct Genomic Proteomic* **4**: 355-362.

Sola-Landa, A., R. S. Moura & J. F. Martin, (2003) The two-component PhoR-PhoP system controls both primary metabolism and secondary metabolite biosynthesis in *Streptomyces lividans*. *Proc*

Natl Acad Sci U S A **100**: 6133-6138.
Sola-Landa, A., A. Rodriguez-Garcia, A. K. Apel & J. F. Martin, (2008) Target genes and structure of the direct repeats in the DNA-binding sequences of the response regulator PhoP in Streptomyces coelicolor. *Nucleic Acids Res* **36**: 1358-1368.
Sola-Landa, A., A. Rodriguez-Garcia, E. Franco-Dominguez & J. F. Martin, (2005) Binding of PhoP to promoters of phosphate-regulated genes in Streptomyces coelicolor: identification of PHO boxes. *Mol Microbiol* **56**: 1373-1385.
Steinfath, M., D. Repsilber, M. Scholz, D. Walther & J. Selbig, (2007) Integrated data analysis for genome-wide research. *EXS* **97**: 309-329.
Sun, J., A. Hesketh & M. Bibb, (2001) Functional analysis of relA and rshA, two relA/spoT homologues of Streptomyces coelicolor A3(2). *J Bacteriol* **183**: 3488-3498.
Szeszak, F., S. Vitalis, F. Toth, G. Valu, J. Fachet & G. Szabo, (1990) Detection and determination of factor C--a regulatory protein--in Streptomyces strains by antiserum and monoclonal antibody. *Arch Microbiol* **154**: 82-84.
Tahlan, K., S. K. Ahn, A. Sing, T. D. Bodnaruk, A. R. Willems, A. R. Davidson & J. R. Nodwell, (2007) Initiation of actinorhodin export in Streptomyces coelicolor. *Mol Microbiol* **63**: 951-961.
Takano, E., R. Chakraburtty, T. Nihira, Y. Yamada & M. J. Bibb, (2001) A complex role for the gamma-butyrolactone SCB1 in regulating antibiotic production in Streptomyces coelicolor A3(2). *Mol Microbiol* **41**: 1015-1028.
Takano, E., H. C. Gramajo, E. Strauch, N. Andres, J. White & M. J. Bibb, (1992) Transcriptional regulation of the redD transcriptional activator gene accounts for growth-phase-dependent production of the antibiotic undecylprodigiosin in Streptomyces coelicolor A3(2). *Mol Microbiol* **6**: 2797-2804.
Tan, H., H. Yang, Y. Tian, W. Wu, C. A. Whatling, L. C. Chamberlin, M. J. Buttner, J. Nodwell & K. F. Chater, (1998) The Streptomyces coelicolor sporulation-specific sigma WhiG form of RNA polymerase transcribes a gene encoding a ProX-like protein that is dispensable for sporulation. *Gene* **212**: 137-146.
Tanaka, A., Y. Takano, Y. Ohnishi & S. Horinouchi, (2007) AfsR recruits RNA polymerase to the afsS promoter: a model for transcriptional activation by SARPs. *J Mol Biol* **369**: 322-333.
Tang, Y. J., H. G. Martin, S. Myers, S. Rodriguez, E. E. Baidoo & J. D. Keasling, (2009) Advances in analysis of microbial metabolic fluxes

via (13)C isotopic labeling. *Mass Spectrom Rev* **28**: 362-375.
Tiffert, Y., P. Supra, R. Wurm, W. Wohlleben, R. Wagner & J. Reuther, (2008) The Streptomyces coelicolor GlnR regulon: identification of new GlnR targets and evidence for a central role of GlnR in nitrogen metabolism in actinomycetes. *Mol Microbiol* **67**: 861-880.
Tomono, A., M. Mashiko, T. Shimazu, H. Inoue, H. Nagasawa, M. Yoshida, Y. Ohnishi & S. Horinouchi, (2006) Self-activation of serine/threonine kinase AfsK on autophosphorylation at threonine-168. *J Antibiot (Tokyo)* **59**: 117-123.
Traag, B. A., G. H. Kelemen & G. P. Van Wezel, (2004) Transcription of the sporulation gene ssgA is activated by the IclR-type regulator SsgR in a whi-independent manner in Streptomyces coelicolor A3(2). *Mol Microbiol* **53**: 985-1000.
Traag, B. A. & G. P. van Wezel, (2008) The SsgA-like proteins in actinomycetes: small proteins up to a big task. *Antonie Van Leeuwenhoek* **94**: 85-97.
Tunca, S., C. Barreiro, J. J. Coque & J. F. Martin, (2009) Two overlapping antiparallel genes encoding the iron regulator DmdR1 and the Adm proteins control siderophore [correction of sedephore] and antibiotic biosynthesis in Streptomyces coelicolor A3(2). *FEBS J* **276**: 4814-4827.
Tunca, S., C. Barreiro, A. Sola-Landa, J. J. Coque & J. F. Martin, (2007) Transcriptional regulation of the desferrioxamine gene cluster of Streptomyces coelicolor is mediated by binding of DmdR1 to an iron box in the promoter of the desA gene. *FEBS J* **274**: 1110-1122.
Uguru, G. C., K. E. Stephens, J. A. Stead, J. E. Towle, S. Baumberg & K. J. McDowall, (2005) Transcriptional activation of the pathway-specific regulator of the actinorhodin biosynthetic genes in Streptomyces coelicolor. *Mol Microbiol* **58**: 131-150.
UHNMEE, #160, P., SUH, J.-W., HONG & S.-K., (2000) *Genetic analysis of absR, a new abs locus of Streptomyces coelicolor*. Korean Society for Applied Microbiology, Seoul, COREE, REPUBLIQUE DE.
Umeyama, T. & S. Horinouchi, (2001) Autophosphorylation of a bacterial serine/threonine kinase, AfsK, is inhibited by KbpA, an AfsK-binding protein. *J Bacteriol* **183**: 5506-5512.
Umeyama, T., P. C. Lee, K. Ueda & S. Horinouchi, (1999) An AfsK/AfsR system involved in the response of aerial mycelium formation to glucose in Streptomyces griseus. *Microbiology* **145 (Pt 9)**: 2281-2292.
van Wezel, G. P. & K. J. McDowall, (2011) The regulation of the secondary metabolism of Streptomyces: new links and experimental advances.

Nat Prod Rep **28**: 1311-1333.
van Wezel, G. P., J. van der Meulen, S. Kawamoto, R. G. Luiten, H. K. Koerten & B. Kraal, (2000) ssgA is essential for sporulation of Streptomyces coelicolor A3(2) and affects hyphal development by stimulating septum formation. *J Bacteriol* **182**: 5653-5662.
Velculescu, V. E., L. Zhang, B. Vogelstein & K. W. Kinzler, (1995) Serial analysis of gene expression. *Science* **270**: 484-487.
Vogtli, M., P. C. Chang & S. N. Cohen, (1994) afsR2: a previously undetected gene encoding a 63-amino-acid protein that stimulates antibiotic production in Streptomyces lividans. *Mol Microbiol* **14**: 643-653.
Vujaklija D Fau - Horinouchi, S., T. Horinouchi S Fau - Beppu & T. Beppu, (1993) Detection of an A-factor-responsive protein that binds to the upstream activation sequence of strR, a regulatory gene for streptomycin biosynthesis in Streptomyces griseus. *J. Bacteriol.*
Waldvogel, E., A. Herbig, F. Battke, R. Amin, M. Nentwich, K. Nieselt, T. E. Ellingsen, A. Wentzel, D. A. Hodgson, W. Wohlleben & Y. Mast, (2011) The PII protein GlnK is a pleiotropic regulator for morphological differentiation and secondary metabolism in Streptomyces coelicolor. *Appl Microbiol Biotechnol* **92**: 1219-1236.
Wang, C., X. Long, X. Mao, H. Dong, L. Xu & Y. Li, (2010) SigN is responsible for differentiation and stress responses based on comparative proteomic analyses of Streptomyces coelicolor wild-type and sigN deletion strains. *Microbiological Research* **165**: 221-231.
Wang, C. G., Huanxi; Dong, Huijun; Zhu, Chenggang; Li, Yongquan; Zheng, Jing; Cen, Peilin, (2007) A novel pair of two-component signal transduction system ecrE1/ecrE2 regulating antibiotic biosynthesis in Streptomyces coelicolor. *Biologia* **62**: 511-516.
Wang, X.-J. G., Suo-Lian; Guo, Wan-Qian; Xi, Di; Xiang, Wen-Sheng, (2009) Role of nsdA in negative regulation of antibiotic production and morphological differentiation in Streptomyces bingchengensis. *Journal of Antibiotics* **62**: 309-313.
Wang, Z., M. Gerstein & M. Snyder, (2009) RNA-Seq: a revolutionary tool for transcriptomics. *Nat Rev Genet* **10**: 57-63.
White, J. & M. Bibb, (1997) bldA dependence of undecylprodigiosin production in Streptomyces coelicolor A3(2) involves a pathway-specific regulatory cascade. *J Bacteriol* **179**: 627-633.
Wolanski, M., R. Donczew, A. Kois-Ostrowska, P. Masiewicz, D. Jakimowicz & J. Zakrzewska-Czerwinska, (2011) The level of AdpA directly affects expression of developmental genes in Streptomyces

coelicolor. *J Bacteriol* **193**: 6358-6365.
Xu, D., T.-J. Kim, Z.-Y. Park, S.-K. Lee, S. H. Yang, H.-J. Kwon & J.-W. Suh, (2009) A DNA-binding factor, ArfA, interacts with the bldH promoter and affects undecylprodigiosin production in Streptomyces lividans. *Biochemical and Biophysical Research Communications* **379**: 319-323.
Xu, D., N. Seghezzi, C. Esnault & M. J. Virolle, (2010a) Repression of antibiotic production and sporulation in Streptomyces coelicolor by overexpression of a TetR family transcriptional regulator. *Appl Environ Microbiol* **76**: 7741-7753.
Xu, G., J. Wang, L. Wang, X. Tian, H. Yang, K. Fan, K. Yang & H. Tan, (2010b) "Pseudo" gamma-butyrolactone receptors respond to antibiotic signals to coordinate antibiotic biosynthesis. *J Biol Chem* **285**: 27440-27448.
Xu, W., J. Huang & S. N. Cohen, (2008) Autoregulation of AbsB (RNase III) expression in Streptomyces coelicolor by endoribonucleolytic cleavage of absB operon transcripts. *J Bacteriol* **190**: 5526-5530.
Xu, W., J. Huang, R. Lin, J. Shi & S. N. Cohen, (2010c) Regulation of morphological differentiation in S. coelicolor by RNase III (AbsB) cleavage of mRNA encoding the AdpA transcription factor. *Mol Microbiol* **75**: 781-791.
Yanai, K., T. Murakami & M. Bibb, (2006) Amplification of the entire kanamycin biosynthetic gene cluster during empirical strain improvement of Streptomyces kanamyceticus. *Proc Natl Acad Sci U S A* **103**: 9661-9666.
Yang, Y.-H., J.-N. Kim, E. Song, E. Kim, M.-K. Oh & B.-G. Kim, (2008) Finding new pathway-specific regulators by clustering method using threshold standard deviation based on DNA chip data of <i>Streptomyces coelicolor</i&gt. *Applied Microbiology and Biotechnology* **80**: 709-717.
Yang, Y. H., E. Song, E. J. Kim, K. Lee, W. S. Kim, S. S. Park, J. S. Hahn & B. G. Kim, (2009) NdgR, an IclR-like regulator involved in amino-acid-dependent growth, quorum sensing, and antibiotic production in Streptomyces coelicolor. *Appl Microbiol Biotechnol* **82**: 501-511.
Yang, Y. H., E. Song, B. R. Lee, E. J. Kim, S. H. Park, Y. G. Kim, C. S. Lee & B. G. Kim, (2010) Rapid functional screening of Streptomyces coelicolor regulators by use of a pH indicator and application to the MarR-like regulator AbsC. *Appl Environ Microbiol* **76**: 3645-3656.
Zhang L, L. W., Zhao CH, Chater KF, Tao MF., (2007) NsdB,a TPR-like-domain-containing protein negatively affecting production of antibiotics in Streptomyces coelicolor A3(2). *Wei Sheng Wu Xue*

Bao. **47**: 849-854.
Zhang, W., F. Li & L. Nie, (2010) Integrating multiple 'omics' analysis for microbial biology: application and methodologies. *Microbiology* **156**: 287-301.

Abstract in Korean

국문 요약

그람 양성 토양 미생물인 방선균은 복잡한 세포 분화 메커니즘과 이차 대사물질의 생합성으로 인해 복합적인 전사 조절 네트워크를 가지고 있다. 이차 대사물질 합성에 관여하는 중요한 몇 가지 조절자들에 대해서는 이미 연구가 되어 있으나 여전히 많은 수의 조절자들과 그들 간의 네트워크에 대한 연구가 필요하다. 조절자 간의 계층적인 관계와 보고되지 않은 조절자의 기능에 대해 추론하기 위해 마이크로어레이를 통한 목적 전사 조절자의 발현량을 비교하고 그로부터 알고 있는 조절자와의 새로운 관계에 대해 밝혔다. GEO dataset에서 *absB* 제거 변이주에 대한 시간 별 마이크로어레이 데이터를 얻었고 *absB*의 존재 유무에 따른 전사 조절자들의 발현 양상을 비교하였다. 생체 외 리보누클레아제 활성 분석을 통해 *sco6808*의 전사체가 AbsB에 의해 잘리는 것을 확인하였다. 따라서 어레이 데이터를 이용해 보고 되지 않은 조절자 간의 관계에 대해 확인할 수 있었고 다층적 조절 네트워크에 관한 이해를 좀더 쉽게 할 수 있었다.

항생제 생산 증대를 위해 하나의 조절자를 이용하는 것보다는 두 개의 독립적인 조절자의 조합을 이용하는 것이 더 효과적일 것으로 가정하고 항생제 생산의 활성체인 AfsS를 이용하여 실험하였다. *AfsS* 방해 변이주의 시간 별 마이크로어레이 데이터를 이용하여 전사적으로 *afsS*와 독립적인

조절자를 선택했고 그 조절자는 시그마 인자 F의 길항 조절자 SCO4677과 인산염 수송 시스템 조절자인 SCO4228 (PhoU)이다. 활성체인 AfsS는 과발현하고 억제자인 SCO4677이나 PhoU는 제거하는 형태의 조합으로 세포 내 actinorhodin 생산량이 야생형 균주 대비 약 11 배 (BG4677S), 약 149 배 (BG4228S) 증가하였다.

또한, 조각나 있는 전사 조절 네트워크를 분석하고 확장하기 위해 시간에 따른 일련의 마이크로어레이 실험을 *ndgR* 제거 변이주를 이용하여 수행하였다. 이전 연구에서 NdgR은 DNA 친화 포획법과 질량 분석을 통해 아미노산 합성, 쿼럼 감지, 항생제 생산을 조절하는 global 조절자임을 밝혔다. 마이크로어레이 실험을 통해서 추가로 글루타메이트, 가지사슬 아미노산, 인산염, 메티오닌의 수송, 포스포에놀피루브산 포스포트랜스퍼라제 시스템을 통한 탄수화물 흡수와 글리세롤과 같은 탄수화물 대사, 스트레스 반응과 독성 물질에 대한 내성 등을 조절하는 것을 새로 밝힐 수 있었다. 이러한 NdgR의 역할은 EMSA, 세포 생존 능력 실험, 컨포칼 현미경과 TEM을 이용한 이미지 분석 등을 통해 확인하였다. 시간에 따른 일련의 마이크로어레이 실험을 통해 방선균에서 불완전한 전사 조절 네트워크에 NdgR의 global 조절자로써의 역할에 대한 정보를 추가할 수 있었다.

결론적으로, 여러 방법을 통해 전사 조절 네트워크를 규명하고 항생제 생산 증가를 위한 유용한 방법을 개발할 수 있었다.

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주요어: 방선균, 전사조절인자, 질량분석기, DNA 칩, 조절 네트워크, 항생제 대량 생산